A MANUAL OF EXPERIMENTAL EMBRYOLOGY

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THE UNIVERSITY OF CHICAGO PRESS
CHICAGO ILLINOIS

THE UNIVERSITY OF CHICAGO PRESS, CHICAGO 37
Cambridge University Press, London, N W 1 England
The University of Toronto Press, Toronto 5 Canada

Published September 1942. Fourth Impression 1956. Composed and printed by THE UNIVERSITY OF CHICAGO PRESS, *Chicago, Illinois U.S.A.*

INTRODUCTION

Experimental embryology has achieved a prominent position in modern biology its classical experiments and concepts are, by now an integral part of biological thinking Yet few students of biology have an opportunity to obtain a firsthand acquaintance with its methods and materials This is due partly to the difficulties of providing the embryonic material and partly to the technical difficulties of experimentation on living embryos However, a large number of classical experiments do not require exceptional manual skill and are suitable as classroom experiments Most of the experiments described in the following pages have been made by advanced undergraduate and graduate students in a one semester course which has been offered at Washington University since 1935 One of the main assets of such a course is to bring the student into intimate contact with the living developing organism and the enthusiastic response of the students indicates clearly the demand for such an approach to biology

This *Manual* emphasizes the intrinsic factors of morphogenesis that is origin of form and of organs. It includes regeneration but gives little consideration to histogenesis and growth In the selection of the experiments we were guided by practical considerations. Only those elementary experiments were chosen which do not require a high degree of manual skill and which can be done in the limited time of a three-hour laboratory period. We excluded all experiments which require expensive apparatus such as a micromanipulator Special attention was paid to the development of simple and inexpensive instruments for operations. Only such *living material as can be collected in the field or purchased at relatively* small expense is recommended All experiments were devised in such a way that sectioning of the material is not essential for the study of the results For instance regenerated lenses in amphibian larvae can be studied by making them opaque and thus visible by fixation in formaldehyde chorio-allantoic grafts of limbs can be cleared and stained *in toto* with methylene blue etc.

Another consideration which determined the selection of the experiments was their analytical value that is, their expediency in illustrating

In the Appendix the experiments are arranged in groups according to the technical difficulties involved

important principles of morphogenesis. The theoretical significance of the experiments has been strongly emphasized I believe that a course in an experimental branch of biology not only should acquaint the student with new facts but should strengthen his power of reasoning and his logical acuity as well He should be aware of what an experiment proves and of what it does not prove. Each experiment or group of experiments is preceded by a brief outline of its theoretical implications. These general remarks integrate the different problems handled in the *Manual*, but they are not to be considered as a substitute for lectures or for textbooks. On the contrary it is hoped that they will stimulate collateral reading The bibliographies serve the same purpose. They are not complete but give references only to those articles which are directly related to the experiment under consideration and to pertinent review articles. A special chapter on gastrulation in amphibians was added because familiarity with this phase of development is indispensable for experimental work on early embryos

The experiments are organized according to a logical plan This is not necessarily the sequence in which they should be taken up in the laboratory The planning of the course work will depend largely on the availability of living material To facilitate a flexible schedule each experiment has been treated as a separate unit. The technical procedures are described for each experiment separately they are not based on previous experience with other experiments. It is immaterial whether one starts with amphibians, with regeneration in planarians or with chick experiments The selection and arrangement of the exercises is left to the discretion of the instructor A tentative schedule for a one-semester course will be found in the Appendix

The instructions for technical manipulations are given in great detail I hope that they will be useful not only for students but also for research workers in biology experimental medicine, and related fields who may find one or another of the techniques applicable to their own problems The technical procedures have worked satisfactorily in our course which does not mean that they cannot be further improved I hope that the students will feel encouraged to develop their own initiative and resourcefulness in trying out new experiments and in improving the techniques and instruments

The highly specialized technique of tissue culture has been omitted The role of endocrines in morphogenesis is adequately presented in A E Adams *Studies in Experimental Zoology* (1941) which contains all information necessary for experimentation in this field. Experiments on marine animal eggs are dealt with in Just (1939)

I am indebted to many friends and colleagues Many techniques described in the chapter on amphibians have been worked out in the laboratory of Dr H. Spemann (Freiburg, Germany) with whom I was associated for many years. Dr B H. Willier and Dr Mary Rawles generously made available their experiences with operations on the chick embryo I am much obliged to Drs L G Barth G Frankhauser T S Hall J A Moore and C Parmenter for personal communication of technical procedures. Grateful acknowledgment is made to all students and assistants who helped materially to improve the techniques and to revise the outlines Dr K. Gayer kindly read the manuscript and made many helpful suggestions.

I am grateful to Dr R. G Harrison for his kind permission to publish sketches of his unpublished stage series of *Ambystoma maculatum* These sketches, as well as all other original drawings were made by Miss S E. Schweich Acknowledgment is made to the Wistar Institute Edwards Brothers, Incorporated Akademische Verlags-Gesellschaft and Springer s Verlag for permission to reproduce illustrations

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PART I

EQUIPMENT AND INSTRUMENTS

A. OPTICAL OUTFIT

A low power binocular microscope is indispensable for experimental embryological and regeneration studies Any standard model is acceptable The V base with substage mirror is not necessary for most experiments on the contrary, it is preferable to have the glass stage for the operation dish directly on the working table so that the arms of the operator have optimal support Magnifications ranging between ×6 and ×24 are sufficient for most purposes For illumination any lamp which is mounted on an adjustable support, which has a strong light source and which gives an evenly illuminated field may be used. Precautions must be taken against heating of the operation dish In our laboratory makeshift lamps are in use these consist of a 75 watt bulb mounted inside a tin can and a 500-cc. Pyrex boiling flask filled with water which serves as a condenser and cooler both items are clamped on an ordinary iron support. A Beebe binocular magnifying glass which is worn like a pair of spectacles has been found to be extremely useful on many occasions for instance, in preparing glass needles selecting amphibian embryos, or in hypophysectomy of frogs.

B GLASS INSTRUMENTS[1]

(Instruments 3–8 were designed by Spemann)

Material

glass tubing 6–7 mm. in outer diameter
glass tubing 9–10 mm in outer diameter
glass rod 5–6 mm. in outer diameter
rubber tubing 11–12 mm in outer diameter
rubber tubing 8–9 mm. in outer diameter
rubber caps for pipettes
cover glass
Bunsen burner
iron support
file
diamond pencil[2]

Use soft glass throughout do not use Pyrex.

In using the diamond pencil, scratch only halfway around the rod or tube and then break the pieces apart by gentle pressure. Do not mark around the entire circumference.

1 *Pipettes*—Use both 6–7 mm and 9–10-mm glass tubing Cut pieces of about 8 inches (for 2 pipettes) Heat the middle of the piece over the Bunsen burner roll the piece constantly between your fingers to avoid one-sided melting When the glass is softened pull slowly, holding your hands horizontally until the desired diameter is reached Wide-mouthed and gradually tapering pipettes may best be made over a burner with wing top which gives a broad flame. Cut the pipettes to the desired length with the diamond pencil Hold both openings in the flame to smooth the edges Fit rubber caps *(from medicine droppers) over the wide end* Prepare pipettes of different widths ranging between 2 and 5 mm Prepare several of each kind

2 *Capillary pipettes*—To make capillary pipettes use the following procedure Pull out a pipette with as narrow an opening as possible Cut it off a considerable distance from the tapering region and bend the narrow end into a hook by holding it in the flame Hook the pipette over a ring on an iron support placed near the edge of the table with the pipette suspended over the edge so it will not drop on the table when heated (Fig 1, *h*) Heat an area near the tapering part very gently and cautiously with a low Bunsen burner flame or a microburner When properly heated, the tube will be drawn out into a very fine capillary tube of almost microscopic dimension by the weight of the lower wide part. If too much heat is applied the tube will be pulled apart and drop Even so, it can be saved by placing a container with a layer of cotton on the bottom at a point where the pipette will hit the floor Cut off the fine end with a diamond pencil.

3 *Microburner*—The microburner is a small gas burner used for the preparation of very fine glass needles and other instruments. It is best made of an ordinary injection needle whose pointed end is cut off and whose wide end is fastened in rubber tubing connecting with the gas jet It is mounted in a horizontal position by clamping it on a support or otherwise The optimal length of the flame is 5 mm or less. The size of the flame may be controlled by a clamp on the rubber tubing or directly by the gas jet.

Microburners of glass may be easily made of 6–7-mm tubing Prepare a capillary tube according to section 2 and cut off the narrowest part with the diamond pencil. If the opening is too small, the flame will seal it off by melting the edge The flame must be of blue color a yellow flame indicates the melting of the edge Control the gas supply with a clamp

4 *Micropipette (after Spemann)*—This is a micropipette with a lateral hole (Fig 1 *m*) which is used in transplantation experiments for trans-

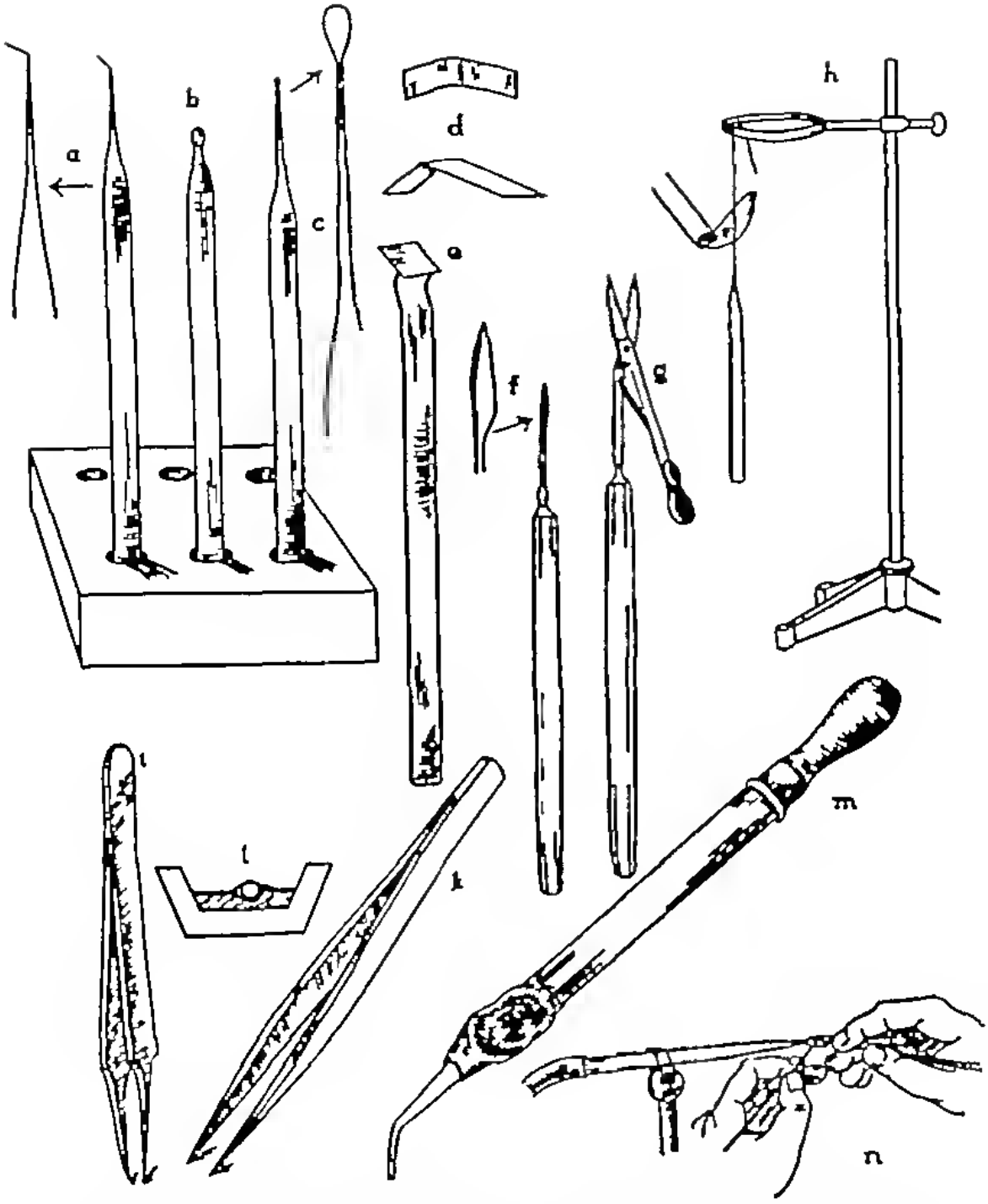

FIG 1.—Instruments for operations on embryos. *a* glass needle *b* glass rod with ball tip *c* hair loop *d* glass bridge *e*, Planaria knife *f* Knapp iris needle *g*, iridectomy scissors *h*, preparation of a capillary pipette *i k* watchmaker forceps *l* operation dish with Permoplast bottom, egg in groove, glass bridge in position *m*, micropipette *n* drawing-out of a glass needle in microburner

ferring small objects. Pull out a fine pipette, about 1–2 mm in narrowest diameter using 7–8-mm glass tubing Make the handle at least 10–12 cm. long To blow a hole in the handle proceed as follows seal the capillary end of the pipette by holding it in the flame Attach a piece of rubber tubing to the wide end of the pipette. Bring one side of the wall of the handle near the point where it tapers very near the flame of a Bunsen burner and heat it thoroughly until it is soft. Take it out of the flame and, while it is hot blow air through the rubber tubing The air cannot escape through the sealed capillary and will blow out the soft glass in the form of a large sausage-shaped, thin walled bubble. The bubble usually explodes instantly, or it can be easily removed Scratch the circumference of the hole with a diamond pencil and break off carefully all glass particles up to this mark Smooth the edge of the hole over the flame Cut off the closed capillary be sure to make a *straight* cut. It is practical to have the capillary opening slightly bent Hold it horizontally over the flame of the microburner in such a way that the hole faces you and the capillary end points to your left. Gently heat the lower wall of the capillary near its opening The end piece will then bend by its own weight With a forceps bend it farther, until it is at an angle of about 120° to the straight part Next cover the hole with a rubber membrane Cut a piece of rubber tubing which will cover the lateral hole, moisten the pipette and the rubber piece and slip the latter over the hole. It must fit very tightly Fit a rubber cap over the wide end

Use of the micropipette —Place your right thumb on the rubber membrane over the hole. Draw in water using the distal cap until the narrow end is filled almost to the level of the hole While the opening is under water, squirt out a small amount of water by gentle pressure on the membrane over the hole Retain the pressure and under the binocular microscope place the opening of the pipette over the object Then release the pressure slowly The object will be drawn into the capillary portion and can be transferred. A slight pressure on the membrane suffices to press it out again

A good micropipette must hold water in the capillary end i e. no water must drip out when it is held with the narrow opening pointing downward

5 *Glass needles* —These needles (Fig 1 *a*) are the universal cutting instruments for extirpation and transplantation experiments on amphibian and chick embryos They are best prepared in two parts First, make 6–10 handles using a glass rod 5–6 mm in diameter Cut pieces about 20 cm long (for 2 handles) Draw them out in the flame of a Bunsen burner break the 2 handles apart, and pull each a second time on the

microburner to obtain a fine point near the tapering end of the handle Next pull out a piece of glass rod to an extremely fine elastic thread much thinner than a hair To do this heat a point and pull the ends apart very quickly Prepare a number of such threads of slightly different thickness Break off a short piece of thread and hold it with your left hand Take the handle in your right hand and bring one end of the thread to the pointed end of the handle, making contact at an angle of about 120° In this position move them slowly toward the microburner (Fig 1, *n*) At the moment when they are heated and fused either pull them apart with a sudden jerk or move them apart slowly The thread will thus be spun out to an exceedingly fine hair Under the binocular microscope clip the end portion near the handle with a watchmaker forceps It should have a pointed end and should be quite elastic. A Beebe binocular magnifier is of great service in preparing needles.

Prepare 6–10 needles to have a supply in case one breaks during an operation. They must be tried out on the object. Most difficulties in operating result from inadequate instruments needles may be too thick or too thin too long or too short too elastic or not elastic enough. Different stages or different types of eggs require slightly different needles.

6 *Hair loop*—The hair loop (Fig 1 *c*) is used for handling living embryos or embryonic tissue Prepare a number of fine capillary tubes as in section 2 Cut their ends off with the diamond pencil. These are the handles They should be about 12 cm long Obtain very fine soft human hair That of babies is best suited the hair of most adults is too coarse. Cut pieces about 3 cm long Hold the handle in your left hand and pick up a piece of hair with a watchmaker forceps which is held in your right hand Under the binocular microscope work first one end of the hair and then the other into the capillary opening of the handle This opening should not be much wider than the diameter of the two hair ends. Push one end deeper into the capillary until the loop has the desired length 3–4 mm The hair must then be sealed into the capillary Melt a small piece of paraffin on a glass plate (slide) and dip the hair loop into it A small amount of liquid paraffin will be sucked into the end of the tube by capillary force and will harden instantly Sometimes a film of paraffin will remain in the hair loop To remove it warm a piece of filter paper on a slide and touch the hair to the warm paper avoid melting the paraffin in the handle Prepare a stock of hair loops of different sizes

7 *Glass rods with ball tips*—Glass rods with ball tips (Fig 1 *b*) are used for making grooves in the Permoplast or paraffin bottom of operation dishes. Pull out a glass rod 5–6 mm in diameter break its slender part near the handle and hold the point downward in the flame of a

microburner (sec 3) It will melt and form a ball The ball should not have a narrow neck because it will break easily Prepare balls of different sizes [3]

8 *Glass bridges* —Glass bridges (Fig 1, *d*, *l*) are used in transplantations of amphibian embryos to hold the transplant in position until it is healed in With diamond pencil and ruler cut a cover glass into small strips 3–4 mm wide. Cut each strip into small rectangular pieces 10–12 mm long Take up each piece with a watchmaker forceps and pull its four edges slowly through the microburner so that all sharp and rough projections disappear All edges must be absolutely smooth. Grasp each glass piece on one narrow end and hold it in a slanted position over the microburner in such a way that an area a short distance from the other end is heated from below The end opposite to the forceps will then bend down by its own weight The glass bridge, when finished, must stand firmly on its narrow edges

The size and angle of the glass bridge must be adapted to the material The glass bridge can be easily cleaned and sterilized by pulling it quickly through a Bunsen burner Prepare 6–8 bridges of slightly different angles and lengths

C METAL INSTRUMENTS

1 *Scalpels, scissors, and forceps* —Scalpels scissors and forceps of different sizes will be needed for many manipulations Stainless-steel instruments are preferable Chromium plated instruments are not recommended because particles of the plating are liable to chip off when one tries to resharpen the instruments.

2 *Watchmaker forceps* —These forceps (Fig 1 *i k*) are universal tools and indispensable for work on embryos. They must have very *fine points* and must work very smoothly that is they must respond to the slightest pressure Instruments made of high grade steel (stainless if possible) should be selected Two types of tweezers are on the market—one with gradually tapering ends and one with shoulders The latter usually have finer points. However any tweezers must be sharpened before use and the tips ground to the finest points Tweezers may be purchased from any wholesale watchmaker or jeweler tool company or through Clay Adams Company (44 East Twenty third Street New York City) The

[3] Glass needles, rods with fine tips, and hair loops may be best mounted on a *wooden holder*—a piece of solid wood, about 20 × 7 × 3 cm with two rows of holes slightly larger than the diameters of the handles and bored at a distance of about 3 cm from one another (Fig. 1 left upper corner)

best instruments are imported from Switzerland (Dumont fils Arrow Royal) They are superior to the imitation makes however the latter are entirely satisfactory for classroom experiments *Sharpening* is best done on an oilstone or most efficiently with a high speed hand tool with replaceable emery wheels *Rust* is best removed with a paper towel and kitchen cleanser

3 *Iridectomy scissors*—These scissors the finest on the market, are used by many investigators for operations on amphibian and chick embryos. For certain types of operations they are preferable to glass needles—they are more durable and they cut in clean straight lines. However their high price prohibits their use as instruments for the classroom and all operations described in this *Manual* were devised for the glass-needle technique Three types of iridectomy scissors are on the market in the De Wecker scissors and the McClure scissors the blades are closed by pressure on both handles in the third type (Fig 1 *g*) one blade is a direct continuation of the long handle and is fixed in position the cutting being done by pressure on the very short handle of the other (movable) blade All three types may be obtained through Clay Adams Company and are illustrated in its catalogue They are equally usable as far as our experience goes

4. *Iris knives*—These knives are the finest steel knives that are on the market They are required for delicate operations in which the smallest ordinary scalpels prove to be too coarse A number of iris and cataract knives of different types are in use in ophthalmology We prefer the single-edged straight Knapp iris needle (Fig 1 *f*) with a half spear point. For use in the classroom we advise preparing fine knives by grinding and sharpening ordinary dissecting needles or sewing needles The latter can be easily mounted in a piece of glass tubing by heating one end of the tubing and introducing into it the eye end of the needle

5 *The Planaria knife* (*after R. Silber*)—The 'planaria knife (Fig 1 *e*) has been found to be very useful in cutting experiments on planarians It consists of a piece of razor blade mounted on a glass handle It is prepared in the following way break a double-edged razor blade in two and break off small pieces of the sharp edge Clean them thoroughly and remove all grease with acetone or alcohol. Cut pieces of 7 mm. glass tubing 10–12 cm long heat one end until it is very soft and press it against a metal plate at an angle of 45° Clean this oblique surface carefully with acetone Glue a chip of razor blade on this surface with a high grade liquid waterproof glue (e g DuPont waterproof transparent cement) Allow the sharp edge to project a few millimeters beyond the handle

elaborate precautions. We suggest the following simple device for their sterilization Slip a pencil clip over the handle of the hair loop or glass needle and fasten it on the edge of a beaker or wide mouthed specimen bottle, with the needle or loop dipping into 70 per cent alcohol

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PART II

EXPERIMENTS ON AMPHIBIAN EMBRYOS

A. LIVING MATERIAL

1 AMPHIBIANS COMMONLY USED FOR EXPERIMENTAL WORK AND THEIR BREEDING HABITS

Most experimental work on amphibian embryos is being done on a few common native forms namely, 3 species of salamanders (*Ambystoma*) 3 newts (*Triturus* one of them *Tr pyrrhogaster* is imported from Japan) and 4 frog species Only these forms will be briefly described in the following paragraphs. Other more rare forms which may be abundant locally, would, in all probability serve many experimental purposes as well However one can by no means suppose a priori that two even closely related forms will behave alike in a given experiment. The differences which were found between related anurans with respect to lens induction (p 107) and between different urodeles with respect to Wolffian lens regeneration (p 176) may serve as a warning To avoid experimental failures due to the choice of unfit material we have indicated for each experiment which species are recommended Those listed are without exception the forms used either by the original investigator or by the author in classroom experiments.

Most of the data for the eastern forms are taken from the monographs of Bishop (1941) for *Urodela* and of Wright (1914) for *Anura* those for the western forms from Storer (1925) and Twitty (1935) These publications also contain extensive bibliographies Students interested in the life histories and habits of their experimental animals should consult these books and also Wright and Wright (1933) and Noble (1931) For questions of taxonomy see Bishop (1941) Cope (1889) Noble (1931) Steyneger and Barbour (1933) Storer (1925) Keys for the identification of eggs and larvae of eastern urodeles may be found in Bishop (1941) Keys for anuran eggs are to be found in Wright and Wright (1924) for tadpoles in Wright (1929) Keys for the eggs larvae and adults of western species are given in Storer (1925)

ORDER *Urodela*

FAMILY AMBYSTOMIDAE

1 *Ambystoma maculatum* (Shaw) frequently referred to as *A punctatum* Spotted salamander Average size 170 mm Color deep bluish

The spelling Ambystoma" has been adopted in this *Manual* following the rules of nomenclature Most experimental embryologists use the traditional spelling "Amblystoma." There

black, with two irregular rows of rounded yellow spots on the back Ventral side lighter The most common *Ambystoma* Widely used for experiments

Breeding places Woodland ponds and slowly running streams.

Breeding season Varies considerably with latitude from January (in southern regions) to May (in northern regions) The breeding season in a given locality lasts for several weeks The animals spend the rest of the year in burrows or under stones and logs on land

Egg masses 100–150 eggs, each in its own jelly membrane are held together in a common jelly envelope, which is firm and globular in shape. They are attached to sticks or float freely, within 8–10 inches from the surface.

Range 'Nova Scotia w to Wisconsin and s. to Georgia and Texas" (Bishop 1941 p 130)

2 *Ambystoma tigrinum* (Green) Tiger salamander This is the largest native *Ambystoma*, averaging 200 mm Deep-brown to black on dorsal side olive-yellow on ventral side Markings dorsally and laterally are irregular olive brown to brownish yellow blotches much duller and more irregular than in *A maculatum*

Breeding places Permanent or temporary ponds

Breeding season In all regions slightly earlier than *A maculatum* During the rest of the year the animals are on land, hidden under stones, logs etc

Egg masses Contain only 30–50 eggs the common jelly envelope is softer less firm than in *A maculatum* They are usually fastened to twigs and branches 12 inches or more under the surface

Range ' From Long Island s to n. Florida w to Mississippi and Arkansas and n to Minnesota and Ontario (Bishop 1941 p 172)

3 *Ambystoma opacum* (Gravenhorst) Marbled salamander The smallest of the three about 100 mm Color black with light markings on the back and on the sides Their color is dull gray in the ♀, and bright in the ♂ very variable in size and shape.

Breeding places This form does not lay its eggs in the water but hides them in shallow grooves under leaves or under logs in woods. The breeding places are on the margin of temporary ponds or swampy places which are dry at the breeding season but which will be submerged later in the

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can be no doubt that the latter is linguistically correct, meaning blunted mouth," whereas the one which enjoys priority is meaningless and is probably a typographical error I agree fully with Dr Harrison's characterization of this situation (in Jour Exper Zool 41:351 n)

year Many females migrate to the same place so that one can usually collect from many nests in the same location.

Breeding season September and October

Egg masses The eggs are laid singly, not held together by a common envelope. Particles usually stick to the surface of the eggs whereby the eggs are well concealed. The membranes will swell and the larvae will hatch when the eggs are placed in water

Range 'From Massachusetts to Georgia w to Louisiana and Texas Mississippi Basin n. to Arkansas Missouri Indiana and Illinois' (Bishop 1941 p 154)

FAMILY SALAMANDRIDAE

4. *Triturus viridescens* Rafinesque Common newt Average size 85 mm Color in the aquatic form dorsal side olive-green ventral side light to bright yellow Small black spots scattered over both sides A series of black spots on either side of the dorsal midline The land form called eft is bright red. During the breeding season the males can be easily recognized by their broad, wavy tail fins and by the presence of black bars on the ventral surface of the hind legs

Breeding places Ponds lakes and slowly moving waters

Breeding season Variable with latitude April to June and more extended than in frogs and *Ambystoma*

Eggs are deposited singly on leaves of *Vallisneria Elodea* and other aquatic plants. The female grasps a leaf with her hind legs deposits the egg on it and then folds the leaf so that the egg is almost entirely concealed The outermost egg capsule is sticky milky white.

Females will lay eggs readily in the laboratory They should be kept in large aquaria, and plenty of fresh *Elodea* or *Vallisneria* or narrow slips of paper should be provided To insure fertilization a number of males should be added They will court and clasp the females and deposit their spermatophores in the aquarium.

Range N Ontario s to Georgia w to Alabama n. to northern Illinois and Wisconsin (Bishop 1941 p 76)

5 *Triturus torosus* (Rathke) Pacific Coast newt Average size 200 mm and more Dark brown on the upper side orange (or yellow) on the ventral side Surface rough in terrestrial individuals smoother in aquatic individuals Common

Breeding places Creeks and ponds

Breeding season Varies with latitude January and February in low altitudes until early summer in high altitudes

Egg masses 10–25 eggs are laid simultaneously they stick together

and form a firm clump but have no common envelope They are attached to water plants

Range All California, from San Diego to Alaska

Twitty has discovered two new species of *Triturus* in California *Tr simulans* and *Tr rivularis* Both have been used for experiments (Twitty 1935, 1936)

ORDER *Anura*

FAMILY RANIDAE

6 *Rana pipiens* Schreber Leopard frog Average length 80–90 mm Dorsal side with rounded or oval dark spots rather irregularly spaced Smaller spots on the sides Ventral side whitish or yellowish The males can be easily recognized by the swellings at the thumbs The commonest and most widely used frog

Breeding places In ponds and swampy marshlands.

Breeding season Early spring (March and April) The adults spend the summer on land and hibernate in the water, hidden beneath stones or logs

Egg masses Contain approximately 3 000 eggs. The jelly membranes of the individual eggs stick together but they are not inclosed in a common envelope. They are laid near the surface usually a considerable number of masses are deposited at the same place

Range Most parts of the United States, except the Pacific Coast.

7 *Rana palustris* LeConte The pickerel frog Average size 70 mm smaller than *R pipiens* Upper side pale brownish with dark spots these are larger and more regularly arranged than those of *R pipiens* namely, in two distinct rows They are oblong or square Underside yellowish white *R palustris* can be easily distinguished from *R. pipiens* by the bright yellow color of the underside of the thighs Males have thickened thumbs.

Breeding places Cold springs and streams, ravines ponds

Breeding season Late April and May The adults spend the summer in marshy places ravines moss bogs etc. and hibernate in water covered by logs and stones

Egg masses Contain about 2 000 eggs They are laid in shallow water and are usually attached to sticks The masses are much like those of *R pipiens* but the eggs can be easily recognized by the brown animal pole and the yellow vegetal pole in contrast to the black and white of *R pipiens*

Range N to Canada W to Great Plains S to Louisiana and Florida

8 *Rana sylvatica* LeConte. Wood frog The smallest of the common

frogs, 65 mm Upper side, gray to brownish with dark streaks on both sides of the head and a few scattered dark spots. Lower side whitish Males have swollen thumbs

Breeding places Still waters ponds, transient pools in woods.

Breeding season Slightly earlier than the other common frogs, late March and April as soon as the ice has left. The frogs spend the summer in the woods and hibernate on land, in woods under cover

Egg masses Contain about 2 000 eggs. They are similar to those of *R. pipiens* but more globular and individual eggs less crowded i e , outer jelly membranes thicker Most frequently attached to water plants twigs etc.

Range N to Maine s to North Carolina w to Missouri

9 *Rana catesbeiana* Shaw Bullfrog The largest of all native frogs up to 17–20 cm Upper side brown ventral side, white. Males have thickened thumbs

Breeding places Lakes ponds brooks marshy swamps, streams. The adults stay in the water throughout the year The tadpoles spend two years in the larval stage

Breeding season Later than most other frogs June and July in northern parts somewhat earlier in southern parts

Egg masses Contain more eggs than those of other frogs 10 000 and more being reported They are laid as a surface film The jelly is loose and gelatinous and less compact than that of the other common frogs. The egg masses are deposited among brush at the edge of the ponds

Range Eastern states to the Rocky Mountains

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2 STAGE SERIES RATES OF DEVELOPMENT AND OTHER DATA

For practical purposes it is desirable to break up the continuous process of development into discrete "stages" and to agree on standardized "stage series" for convenient reference in descriptive and experimental work. The stages should be characterized by easily identifiable external features In cold blooded animals stage seriations in terms of age are useless because the rate of development varies with temperature It should be understood that, in the stage series listed below the time intervals between any two successive stages are not constant but differ from stage to stage The series for different forms have been worked out by different authors, and their initials will be added to the stage numbers. This practice will be followed throughout this *Manual*

TABLE 1

TIMETABLE FOR *A maculatum*

(At Room Temperature, Approx. 20 C)

	Stages											
	H	H_3	H_4	H_5	H_7	H_9	H	H_{3}	H_{4}	H_{17}	H_{9}	H_{3}
Time	0	2	4	6	12–14	34–36	2	2½	3	3½	3½–4	4½–5

H_3–H_9 in hours from H 1 on, in days

	Stages										
	H_{29}	H_{34}	H_{36}	H_{38}	H_{39}	H_4	H_4	H_{43}	H_{44}	H_{45}	H_{46}
Time	7	8	9	12	14	15–16	18–19	20	21	23–24	25

ORDER *Urodela*

1 *Ambystoma maculatum*.—Harrison s excellent series for this widely used salamander is the most complete and most perfect stage series devised so far and should be used as a model for any other urodele series. It is not published, but it is widely circulated through the courtesy of the author and generally adopted by experimental embryologists. It covers with 46 stages the period from the uncleaved egg to the stage in which the larva begins to feed The stages are depicted in Figure 45

Rate of development —This rate varies with temperature and other external factors Table 1 is compiled for practical purposes The data are combined from Dempster (1933) J Moore (1939) and my own notes all three of which are in close agreement

OTHER DATA ON NORMAL DEVELOPMENT

Rupture of vitelline membrane Late neurula stage
First movements Stages H32–H34 (for details see p 124)
First heartbeat H34.
Hatching H40–H42 = 15–19 days
Beginning of feeding H45–H46 = 25 days
Metamorphosis Varies considerably with feeding etc from 70 days (at maximal feeding Twitty and Schwind 1931) to 120 days or more
Growth curves May be found in Harrison (1929) Stone (1930) Twitty and Schwind (1931) Dempster (1933) Moore (1939) At metamorphosis the animals have attained a length of 48–55 mm
Volumetric and dry-weight measurements See in Dempster (1933)
Density measurements See in Brown Hamburger and Schmitt (1941)
Temperature tolerance From 3 5° to 23 C (Moore 1940*b*)

2 *Ambystoma tigrinum* —No stage series for this form has been worked out The H-stages for *A maculatum* can be applied roughly to this form if one uses gill and tail development for identification but disregards limb development. The forelimb buds which appear in *A maculatum* in Stages H36–H37 appear in *A tigrinum* very belatedly namely in the feeding stage which corresponds to H46 for *A maculatum* Another profound difference between the two forms is found in their *growth rates* Adults of *A tigrinum* are of approximately double the size of *A maculatum* and this difference is reflected in the higher growth rate (increment in length per time unit) of *A tigrinum* from early stages on Growth curves for *A tigrinum* are to be found in Harrison (1929) Stone (1930) Twitty and Schwind (1931) Moore (1939) As a result of the higher growth rate maximally fed larvae of *A tigrinum* are about 100–110 mm long at metamorphosis as compared to 48–55 mm for *A maculatum* Both forms if fed maximally metamorphose at about the same time (approximately 76 days after fertilization according to Twitty and Schwind 1931) Under less favorable conditions the time of metamorphosis of *A tigrinum* is extremely variable it may be delayed up to 17 months (Harrison 1929) Stone (1930) gives 130 days as an average

The rapid rate of development of *A tigrinum* is also expressed in the

Du Shane and Hutchinson (1941 p 237) indicate that in all probability genetic differences in developmental rates exist between eastern and middle western races.

greater speed with which a given H stage is reached, as compared to *A maculatum* According to Moore (1939) and my own limited data, the rates of development compare approximately as shown in Table 2

Hatching takes place in approximately the same stage as in *maculatum*, which is the equivalent of H40–H42 (13–14 days) However the fore limbs, at that stage are barely visible buds they are comparable to those of H37 for *A maculatum*

3 *Ambystoma opacum* —No stage series has been worked out Growth proceeds more slowly even than in *A maculatum* Growth curves in Twitty and Elliott (1934 p 284) and Moore (1939)

Density measurements See in Brown Hamburger, and Schmitt (1941) and Brown (1942)

TABLE 2

DAYS REQUIRED TO REACH A GIVEN H STAGE

(At Approx 20 C)

	Stages					
	H	H 3	H3+	H39	H41	H46
A tigrinum	1½	3	5–6	8–9	11–12	15 16
A maculatum	2	4	7–8	14	16–18	25

4 *Triturus* —No stage series have been published for the American species A stage series of *Tr pyrrhogaster* was published by Oyama (1930) several stages are described in Yamada (1939) Concerning European newts the series of Glässner (1925) for *Tr vulgaris* (*taeniatus*) is not satisfactory because the stages are too far apart and the schematic drawings often make a diagnosis difficult Sato (1933) gives a clearly defined and well illustrated seriation of the tail bud stages of *Tr vulgaris*, which are numbered in conformity with Harrison s stages (H21–H32) Glücksohn (1931) has worked out complete seriations for the larval periods of *Tr vulgaris* and *Tr cristatus* the most commonly used European newts Her seriation begins with a stage corresponding to H36 and ends with metamorphosis (Stage 62 for both forms) As criteria for identification the development of forelimbs and hind limbs and in particular the relative sizes of toes are used The numbering is adapted to the H series A seriation for another widely used European newt *Tr alpestris* was worked out by Knight (1938)

5 *Rana pipiens*—A stage seriation has been published by Shumway (1940) (see Fig 43) It covers the phase from fertilization to the young tadpole in which the gills are just overgrown by the operculum and the animal has not fed yet Twenty five stages are distinguished. They are conveniently tabulated, together with average body length and age (at 18° C) This seriation is already widely adopted and will be referred to in the text as Sh1–Sh25

Rate of development—The studies of Atlas (1935) and of Moore (1939) give full information on this point (see also Shumway 1940) In Moore's paper the rate of development (in hours) in terms of Sh-stages is given for four different temperatures (15 3° 18 6° 19.8° 26° C) The following table includes Moore's figures for room temperature 19 8° C

TABLE 3

RATES OF DEVELOPMENT FOR FOUR SPECIES OF *Rana*

(Time in Hours after First Cleavage at 19 8° C., from Moore 1939)

Stage	*R. pipiens*	*R. palustris*	*R. sylvatica*	*R. catesbeiana**
Sh3	0	0	0	0
Sh10	20–24	20–24	16–20	23
Sh11	25	29 5	20–23	
Sh12	29–37 5	35	23–26	
Sh13	38–44	38–43	37 7	42
Sh14	43 5–48	46–55	37 7	
Sh15	50–54 5	55	40 5	60
Sh16	51–58	61–64	40–45	
Sh17	60–69	66–74		72
Sh18	70–84	80–83	50	
Sh19	85–96	95–98	66	
Sh20	95–103	105–106	72–87	157

* Personal communication of Dr John A Moore

OTHER DATA ON NORMAL DEVELOPMENT

First movements Sh18

First heartbeat Sb19

Hatching Sh18 (Moore 1940) Sh20 (Shumway 1940)

Spontaneous swimming Sh21

Feeding Sh25

Metamorphosis in the field July i.e., 13–16 weeks after egg laying (Wright 1914)

Temperature tolerance 6°–38° C (Moore 1939 1942)

Density measurements See in Brown Hamburger and Schmitt (1941)

6 *Rana sylvatica*—In the stage series for this form by Pollister and Moore (1937) the period from the uncleaved egg to the beginning of the

overgrowth of the operculum is divided into 23 stages comparable to Sh1–Sh23 for *R. pipiens*[1] They will be designated as PM1–PM23 See Figure 44

Rate of development—It is considerably faster than that of *R. pipiens* See Table 3

OTHER DATA ON NORMAL DEVELOPMENT

First motility PM18

First heartbeat PM19

Hatching PM20–PM21

Spontaneous swimming PM23

Metamorphosis in the field Early in July that is 14–16 weeks after egg laying (Wright 1914)

Temperature tolerance The eggs of this frog which is the earliest breeder (middle of March) can tolerate a lower temperature than others but they are less resistant to higher temperatures The range of tolerance is from 2 5° to 24° C (Moore, 1939 1942)

7 *Rana palustris*—No stage series is available.

Rate of development—At 19.8° C see Table 3 For rates for other temperatures see Moore (1939) The development is somewhat slower than that of *R pipiens*

Hatching Stage PM17–PM18

Metamorphosis in the field In August, that is 14–17 weeks after egg laying (Wright 1914)

Temperature tolerance From 7°–30° C (Moore 1939, 1942)

8 *Rana catesbeiana*—No stage series is available. The eggs are laid late in the season and metamorphosis takes place two years later

Rate of development See Table 3

Hatching Stages PM17–PM18

Temperature tolerance 15°–32° C (Moore, 1942)

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[1] Shumway's stages were adapted to those of Pollister and Moore which were published first

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3 CULTURE MEDIA FOR OPERATED EMBRYOS

Most experiments on amphibian embryos require the removal of all jelly membranes previous to the operation. The decapsulated early embryos are very sensitive and must be kept in a sterile salt solution of appropriate osmotic pressure pH etc. From late tail-bud and early swimming stages on embryos are much more resistant and may be kept in tap

water or spring or pond water Amphibian Ringer solution has long been known to be strongly hypertonic. Holtfreter (1931) recommended a dilute Ringer solution which is now generally adopted and known as "Holtfreter solution Its composition is shown in Table 4.

In preparing the sterile Holtfreter solution use distilled water The solution is prepared without $NaHCO_3$ and autoclaved $NaHCO_3$ is sterilized dry and then added to avoid its precipitation

Holtfreter solution is still hypertonic, and amphibian gastrulae will exogastrulate if kept in this medium after removal of the vitelline membrane (Holtfreter, 1933) A 0 6 per cent Holtfreter solution has approximately the correct osmotic pressure. In general a more dilute Holtfreter solution is in use Empirical data show that $\frac{1}{2}$ or $\frac{1}{3}$ or $\frac{1}{5}$ Holtfreter solutions are satisfactory we use $\frac{1}{5}$ throughout with good success. However

TABLE 4

COMPOSITION OF HOLTFRETER AND AMPHIBIAN RINGER SOLUTION

(In Gm /Liter)

Solution	NaCl	KCl	$CaCl_2$	$NHCO_3$
Holtfreter	3 5	0 05	0 1	0 2
Amphibian Ringer	6 5	0 14	0 12	0 1

full-strength Holtfreter solution facilitates healing *per primam* in early embryos and it is a generally adopted practice to do all operations in concentrated Holtfreter solution and keep the embryos in this solution until the wound is healed or the transplant is healed in Then they are transferred to $\frac{1}{2}$ or $\frac{1}{5}$ Holtfreter solution Richards (1940) has compared the properties of the Holtfreter solution with those of the capsular fluid, i.e , the fluid contained in the space between the vitelline membrane and the inner jelly capsule of the egg of *A maculatum* He points out that the Holtfreter solution differs from the capsular fluid not only in its osmotic pressure but also in the following points it lacks protein and probably as a result of this deficiency its viscosity is much lower He suggests adding mucin to the medium However no experiences with this or any other substitute have been recorded

Summary Use concentrated Holtfreter solution for operations and $\frac{1}{2}$ or $\frac{1}{5}$ Holtfreter solution for the rearing of decapsulated embryos up to the swimming stage

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4 REARING AND FEEDING OF LARVAE

REARING OF NORMAL LARVAE

Individual specimens are best kept in Lily cups, Petri dishes etc. For larger cultures large finger bowls or crystallizing dishes of adequate size are recommended (see p 10) Overcrowding should be carefully avoided All tanks should be provided with water plants. Water should be changed whenever necessary, but not too often Allow water to stand for several hours before larvae are placed in it. Tap water in many localities is satisfactory for rearing of older larvae (decapsulated young embryos up to swimming stage should under all circumstances be reared in an artificial medium see p 25) If tap water proves to contain toxic agents, then pond or spring water must be used

Precautions at metamorphosis—Metamorphosis is a critical stage for both anuran and urodele larvae The animals do not feed during this period and become weak unless they have been fed well before the onset of metamorphosis. Facilities for crawling on land must be provided early enough to prevent them from drowning It is best to prepare special tanks for metamorphosis and to transfer into these all old larvae which show signs of metamorphosis (color changes large hind limbs in *Urodela* emergence of one forelimb and tail resorption in *Anura*) In these tanks the water should be only 1–2 inches deep and an incline which rises above the water level should be built either of pebbles or by tilting a glass plate or a glass dish J Moore (personal communication) recommends placing a piece of filter paper on the bottom of a finger bowl tilting the finger bowl and adding a small amount of water

FEEDING OF URODELE LARVAE

Amblystoma and *Triturus* larvae begin to feed in stages corresponding to H46 They are carnivorous and have to be fed live material which moves in front of their eyes and thus attracts their attention Older larvae rely more on their sense of smell and will take small bits of meat Small ostracods, daphnia and other crustaceans are an excellent food for very

small larvae Enchytrae ("white worms —*Enchytraeus albidus*, an oligochaete) are equally suitable and available at all times They may be obtained from fish dealers or pet shops They are best kept in moist humus in a dark, cool room (not warmer than 20 C) and can be cultured easily in the following way Fill a large earthen pot or culture dish with moist humus about 3 inches deep Add a worm culture of at least 2 quarts. Cover it with a lid Feed the worms with white bread cereals or boiled potatoes soaked in milk. Scatter the food about or place it where the worms are congregated and cover it with about an inch of humus. Remove all food particles which begin to decay replace them by fresh food In such cultures worms of all sizes will be found (further details in Blount, 1937)

Special attention should be paid to the feeding of very young larvae when they first begin to eat The very smallest worms of a culture should be selected in the following way Spread humus crumbs and food particles from the worm culture in a Petri dish under water the worms will begin to wriggle vigorously and the smallest ones can be picked out (under the binocular microscope or with the Beebe loupe) At first many larvae are slow and reluctant to take food Yet it is essential to "condition them early to feeding Particularly valuable specimens should be fed individually for a few times. It may be necessary to move a worm slowly in front of the eyes to elicit a snapping reaction As the size increases, larger worms and greater quantities should be fed about every other day Larvae which approach metamorphosis can be fed on small bits of raw liver or beef or pieces of rainworm Precaution must be taken that larvae do not injure one another They will snap at any object that moves and will bite off one another's gills or limbs. Feed the animals abundantly Valuable specimens should be kept in isolation

FEEDING OF ANURAN TADPOLES

The feeding habits of anuran tadpoles are entirely different The tadpoles are equipped with horny teeth and are omnivorous they rasp off algae from the walls of the aquarium or from water plants, they feed on dead animals, meat etc. For rearing of tadpoles Adams (1941) recommends the following beef and wheat mixture

Dissolve one 2-oz (56 68 gm) tube of Bacto-beef extract "Difco" standardized (Digestive Ferment Co Detroit, Mich) in approximately 160 cc. of water Mix wheat flour with this in proportion 1 1 or 2 1 Spread paste thinly on glass plate and dry Grind dried paste to powder with mortar and pestle. Store powder in stoppered bottle

Approximately 2 5 gm of this mixture should be given to each group of 10 animals every day or every other day This food is particularly recommended for controlled feeding (e g., thyroid feeding) experiments A good substitute for this mixture is Pablum Ordinarily algae boiled spinach, boiled meat (finely minced liver beef or frog muscle etc) the yolk of hard boiled eggs or any combination of these will give satisfactory results Tadpoles are voracious eaters and their growth and metamorphosis can be speeded up considerably by heavy feeding Carefully remove all food debris

SOME COMMON DISEASES

Operated animals suffer occasionally from *edema* particularly in tail bud stages. Edematous blebs appear on the flank or on the ventral side Such animals usually die after several days and should be fixed in early stages of edema if they are valuable material. Sometimes puncturing of the vesicle with a fine glass needle saves the animal (narcotize the animal if necessary)

Fungus infections (*Saprolegnia*) are not infrequent in urodele cultures and are difficult to get rid of They appear as fine sticky threads first on gills and legs and eventually all over the body and are usually fatal Detwiler and McKennon (1929) recommend a bath of mercurochrome (di brom-oxy mercuri fluorescin) Animals are kept for several days in a concentration of 1 500 000–1 1 000 000 The molds usually slough off after 2–3 days. Effective concentrations and lengths of exposure should be tried out for each instance

Young urodele larvae which are not properly fed or which are slow in catching food sometimes swallow *air bubbles* Occasionally they fill their stomach with air and float on the surface This condition is of course critical and the larvae will die of starvation unless the air is removed One can accomplish this either by puncturing the stomach with a glass needle or a fine steel knife or by squeezing out the air through the mouth holding the animal cautiously with one pair of watchmaker forceps and pinching behind the air bubble with another forceps.

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B EXPERIMENTS

1 SOME TECHNICAL PROCEDURES

a) EXPERIMENTAL OVULATION BY HYPOPHYSIS INJECTION AND ARTIFICIAL INSEMINATION IN ANURANS

It is now possible to obtain eggs at almost any time of the year owing to our increased knowledge of the control of reproduction by the anterior lobe of the hypophysis. O M Wolf (1929) first reported experimentally induced ovulation in the frog by injection of anterior lobes of the hypophysis R Rugh (1934–41) has worked out the standard technique on which the following directions are largely based

In *Anura* insemination is external Male and female go into amplexus. The eggs are inseminated externally immediately after they have passed through the cloaca The routine procedure of obtaining fertile eggs is to induce ovulation strip the eggs into a sperm suspension and submerge them in water 5 or 10 minutes later to allow the membranes to swell

The following points are of importance for obtaining optimal results.

1 *Source of anterior lobe substance*—So far fresh frog pituitary gland is the only reliable and adequately standardized source for the gonadotropic principle effective in frogs. Implants and extracts of mammalian anterior lobe were unsuccessful (Creaser and Gorbman, 1935, 1939)

2 *Size of frogs* (*Rana pipiens*)—Both donors and recipients must be fully mature Do not use females under 75 mm or males under 70 mm in body length (Rugh 1937*b*)

3 *Condition of frogs*—It is important to use only specimens (for donors and recipients) which are in an excellent condition and which have been recently caught The pituitaries of frogs which are held in room temperature for any length of time starved or otherwise kept under inadequate conditions lose their potency

4 *Temperature*—It is advisable to keep the frogs in a cold room (15 – 20 C) before treatment.

5 *Dosage*—The dosage has been standardized for *R pipiens* implants into *R pipiens* (Rugh 1935*a b* 1941 Moore and Barth unpublished)

Drs John A Moore and L G Barth very kindly made available unpublished data on hypophysis injection and fertilization in *R pipiens* I wish to express my thanks to the authors for permission to include their data in the following account.

The number of pituitaries to be injected varies with the season Rugh found that the pituitaries of mature females are twice as potent as those of mature males. The figures in Table 5 combined from Rugh (1941) and Moore and Barth (unpublished) refer to ♀ glands If ♂ glands are used the doses have to be doubled. Injections during the months immediately following the natural breeding season (April May) are usually unsuccessful.

The size and physiological potency of different hypophyses as well as the responsiveness of individual recipients vary considerably so that the above figures give only approximate values It is therefore advisable to inject two or more females for each experiment or demonstration The effect of injections is cumulative and it is usually possible to bring a 'refractory' female to ovulation by administering a second injection 2 days after a first unsuccessful injection.

TABLE 5

EFFECTIVE DOSES FOR OVULATION (*R. pipiens*)

	September–October	November–December	January–February	March–April
No. of ♀ pituitaries required	6–9	5–6	3–5	2–3

Material

bypodermic syringe 2 cc.
hypodermic needle No 20
dissecting instruments including strong and fine scissors and watch maker forceps
section dishes and watch glasses
finger bowls or Petri dishes
ether
battery jars or aquarium jars with lids or wire coverings to keep the recipients
Holtfreter solution or spring or pond water for dilution of sperm fluid

A Beebe binocular magnifying glass is very helpful in the dissection of the bypophysis

Procedure (for R pipiens)

1 Prepare as many battery or glass jars as there are females to be in

In *R palustris R clamitans* and *R catesbeiana* ovulation has been obtained by injection of *pipiens* pituitaries. The dosage in these cases has not been standardized (Rugh, 1935*b*)

jected. Cover the bottom with water about 1 inch deep Cover the jars with heavy glass lids or wire covering

2 Select two or more females as recipients Hold them ready in a small cage

3 *Narcosis* is not necessary, but it facilitates the manipulation of injection Give a light ether narcosis. Place the frog in a tightly covered glass jar attach to the lid a cotton wad moistened with ether Take precautions that the frog does not touch the liquid ether with its skin

4 *Dissection of the pituitary glands from the donors* (*Fig 2*) —Insert one blade of a strong pair of scissors into the mouth at the angle of the jaws and decapitate the frog by a transverse cut behind the tympanic membrane Be sure not to cut more anteriorly Pith the spinal cord and discard the body Wash the head and remove all blood Turn the head upside down Dissect and clean away the skin of the oral cavity and thus expose the base of the skull Locate the ⊥ shaped parasphenoid bone. Make two cuts through the floor of the skull from the cranial cavity toward the eyes (*ab* and *cd* in Fig 2*a*), by inserting the pointed blade of a fine pair of scissors into the foramen magnum or the spinal canal Do not injure the brain tissue With a pair of forceps carefully deflect the triangular piece of bone, thus exposing the ventral aspect of the brain (Fig 2*b*) Locate the hindbrain the infundibulum and the optic chiasma The anterior lobe of the hypophysis can now be recognized by its pinkish color It is either in its normal position (attached to the infundibulum posterior to the optic chiasma) or quite frequently it will adhere to the deflected bone. Grasp the gland with a pair of fine forceps and place it in ⅓ Holtfreter solution in a watch glass. (Make your first dissections under the binocular microscope or with a loupe) The anterior lobe is pinkish and bean shaped Attached to its anterior straight edge is usually a slender whitish body, the pars intermedia and pars nervosa (Fig 2*c*) Remove this tissue with two pairs of watchmaker forceps Dissect as many glands as are required according to Table 5 (p 31) collect them all in the watch glass

5 *Injection* —The glands are injected entire (maceration results in the loss of some active substance) in 1 or 2 injections Draw the glands into the barrel of the syringe Hold the female which is to be injected in your left hand and insert the needle through the flank skin into the body cavity Push the needle forward under the skin and not medially to avoid injury to the *viscera Inject and* then *withdraw the needle* cautiously while you withdraw pinch the skin at the needle entrance to avoid outflow of hypophysis material To insure that no material is lost

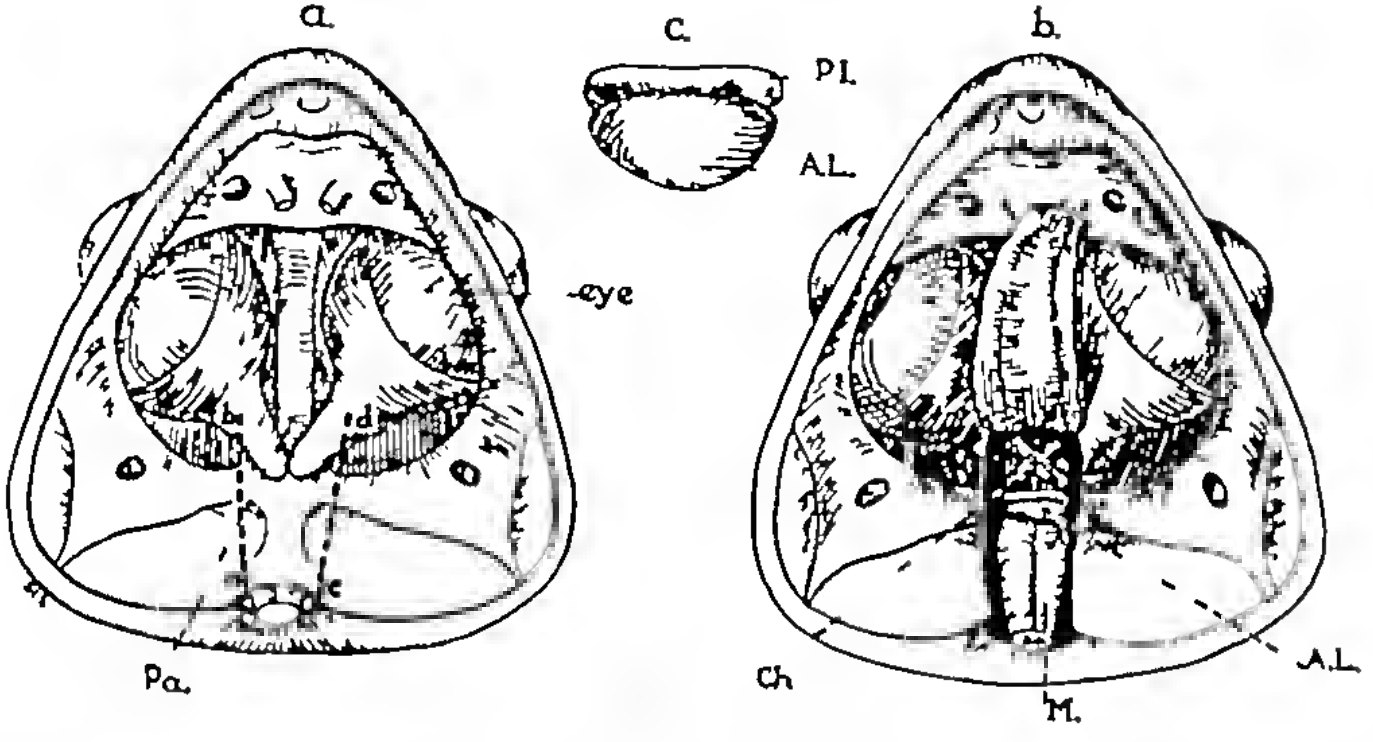

FIG 1.—Hypophysectomy *a*, head of a frog ventral view *Pa*=parasphenoid bone. *b* ventral view brain and hypophysis exposed. *A.L.*=anterior lobe *Ch*=optic chiasma *M*=medulla oblongata. *c* hypophysis isolated. *A.L.*=anterior lobe *P.I.*=pars intermedia.

by adhering to the needle, squirt water through the syringe and inject all material which is recovered in this way

6 Place the female in a jar Label the jar and indicate date and dose. Preferably keep it in a cool place not higher than 20° C

7 *Test of ovulation by "stripping* —At room temperature most of the eggs should have ovulated in 24–48 hours after the injection if the above doses are applied To test ovulation squeeze the female in the following way Bend the legs forward and hold the frog in your right hand. Press gently in the direction of the cloaca In this way eggs will be forced out without injury to the frog If a string of eggs appears, release the pressure at once place the female back in the container, and prepare the sperm fluid. If only liquid or jelly ooze out, then ovulation has not yet taken place, and another test should be made 12 or 24 hours later If no eggs are obtained, a second injection should be made.

8 *Preparation of the sperm fluid* —The males of *R. pipiens* usually contain functional sperm throughout the year so that it is not necessary to give them hypophysis injections. Prepare a dish with 20 cc of 20 per cent Holtfreter solution or 0 1 per cent amphibian Ringer solution or pond or spring water Do not use tap water or distilled water A quantity of 10 cc. per pair of testes is usually recommended for sperm suspensions, but even a tenfold dilution of this suspension still gives optimal results (Moore and Barth unpublished)

Decapitate and pith 2 large males not under 70 mm. in length. Dissect out both pairs of testes (yellow, oval bodies located near the anterior borders of the kidneys) Clean them of adhering blood and tissue and macerate them thoroughly with forceps and scissors, until a milky suspension is obtained. Allow it to stand for 10–15 minutes, during which time the spermatozoa will become active. Observe under the microscope if active motile sperms are present.

9 *Stripping and insemination* —Divide the sperm suspension among 2–3 finger bowls or large Petri dishes so that the bottom of the dish is just covered Hold the female as in section 7 and strip the eggs directly into the sperm fluid by slow continued pressure The eggs will ooze out in a string Line it up in rows or in a spiral, so that all eggs are exposed to the sperm and are not clustered. Shake the dish gently to insure complete fertilization After 5–10 minutes flood the dish with the same medium that was used for sperm suspension rinse and wash the eggs, and let them stand submerged in clean water The jelly membranes will swell slowly A successful insemination is usually indicated after about 1 hour by the rotation of the eggs as a result of which all dark animal poles move upward The first cleavage is to be expected 2½ hours

after insemination If eggs are clustered they should be separated with a scalpel Do not keep more than 30–40 eggs in a finger bowl.

10 *'Fractionated' stripping* —Eggs will remain viable in the oviduct for some time (see below) if the females are kept at a low temperature (10°–15° C) It is therefore possible to obtain eggs from the same female over a period of several days Stripping is simply interrupted when the desired number of eggs is recovered, and the female is returned to the cold room

VIABILITY OF EGGS, SPERM, AND PITUITARY GLANDS (*R. pipiens*)

Eggs —Eggs will remain fully viable and fertilizable and will give an optimal percentage of normal development for 3–4 days after the onset of ovulation if the females are kept at 10°–15° C From then on the percentage of fertilization and of normal development decreases steadily (Zimmerman and Rugh 1941 Moore and Barth, unpublished) Eggs should not be used later than 3 days after ovulation.

Sperm —According to Moore and Barth (unpublished), a sperm suspension of 10 pairs of testes in 150 cc. of 0 1 per cent amphibian Ringer at 15 C retained its fertilizability for 20 hours However it is always advisable to use fresh suspensions

Pituitary glands —According to Rugh (1937*a*) dissected glands retain their potency for a long period if kept in absolute alcohol in the refrigerator Dilute the alcohol to 35 per cent before injection

b) EXPERIMENTAL OVULATION AND ARTIFICIAL INSEMINATION IN *Urodela*

In the *Urodela* the male deposits a spermatophore after a prolonged courtship the female takes it up into a gland of the cloaca (*spermotheca*) where the spermatozoa remain functional for a long period The eggs are inseminated individually while they pass through the cloaca The general practice for obtaining fertile eggs outside of the breeding season is to inject only the females and to rely for fertilization on the presence of functional spermatozoa in the spermotheca The eggs laid by *Triturus viridescens torosus* and *pyrrhogaster* females following hypophysis injection were found to be fertilized in most instances but one occasionally encounters females which lay unfertilized eggs A. E Adams (1930) was the first investigator to obtain experimental ovulation in a urodele *Tr viridescens Triturus viridescens* as well as *R pipiens* hypophyses were used the latter are preferable because they are larger and easier to dissect. *Triturus* females also respond to mammalian pituitary extracts pbyone (a growth stimulation fraction) and hebin (a gonadotropic fraction Adams 1934)

However the dosage has not been standardized We recommend the use of fresh *R. pipiens* glands for *Tr viridescens* (Kaylor, 1937 Griffiths, 1941 Fankhauser, personal communication[1]) The females are kept in the refrigerator at about 10° C between the day of collection and the first implantation This keeps the ovaries in good condition for at least 2 months At room temperature ovaries deteriorate in about 2 weeks. The procedure of injection is the same as in frogs, with slight modifications Whereas in the frog a large number of eggs are ovulated almost simultaneously the eggs of the newts are laid singly over a period of several weeks Therefore in order to obtain a continuous egg production, it is advisable to inject several doses with an interval of 1 or 2 days between the injections. The standard procedure in Dr Fankhauser s laboratory is to inject a single *R pipiens* hypophysis on the first day and another single hypophysis on the third day Two hypophyses were found sufficient to stimulate ovulation at any time between October and May The first eggs are usually laid on the third to sixth day following the first implantation, and the egg laying period lasts between 6 and 14 days on the average. It is advisable to inject a considerable number of females for each experiment, since the number of females which do not respond to the injections or which lay unfertilized eggs is rather high (30–40 per cent) The hypophyses are usually implanted under the skin of the lower jaws. Injected females should be placed in jars or tanks in which they can swim around comfortably and should be amply provided with fresh *Elodea* or *Vallisneria* The eggs are deposited on the leaves of these water plants (see p 17) To collect them it is best to take out all water plants each day and to inspect each leaf Remove the eggs carefully with a watchmaker forceps.

Triturus pyrrhogaster may be treated in the same way (Streett, 1940) In class experiments we obtained from 15 to 150 eggs per female after 2 injections of 2 *R. pipiens* glands each on 2 successive days or with a 1-day interval between the 2 injections

ARTIFICIAL INSEMINATION

Certain experiments for instance hybridization require artificial insemination The eggs cannot be stripped but must be recovered from the oviduct. Prepare a number of Petri dishes laid out with moist filter paper (moist chambers) and clean microscope slides Decapitate and pith several males and females Dissect the testes and the (usually pigmented) vas efferentia and macerate them thoroughly in 10 cc of pond or spring

[1] I am indebted to Dr Gerhard Fankhauser for making available to me his records of injections and for the communication of certain technical details.

water or $\frac{1}{4}$ Holtfreter solution Dissect out the oviducts place them on glass plates and very carefully slit them or cut them open and set the eggs free They are soft and delicate and must be handled with utmost caution Mount them singly on the slides do not moisten them With a fine pipette drop a few drops of the sperm suspension over each egg so that it is well coated Place the slides in the moist chambers for 5–10 minutes then submerge them in water After the membranes are swollen rinse off the sperm suspension and remove the eggs from the slides with a scalpel If artificial hybridization is planned discard all eggs found in the cloaca because they may be fertilized

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c) THE REMOVAL OF EGG MEMBRANES

All amphibian eggs are inclosed in a vitelline membrane and in a number of gelatinous envelopes Their number and consistency differ in dif

ferent species. Descriptions, measurements, and illustrations may be found in Bishop (1941 *Urodela*), Piersol (1929 *A maculatum* and *jeffersoniana*), Wright and Wright (1924, *Anura*)

Ambystoma —The eggs of the three species *A maculatum*, *A tigrinum*, and *A opacum* are approximately equal in size, ranging from 2½ to 3 mm The vitelline membrane is closely applied to the egg A second rather tough and perfectly transparent membrane forms a capsule, about 5½–6 mm in diameter The space between the vitelline membrane and this capsule is filled with the capsular fluid in which the egg moves freely The capsule is surrounded by another tough and less transparent membrane, which consists at least in *A maculatum*, of several thin layers. In *A tigrinum* an additional membrane is found between the two In all

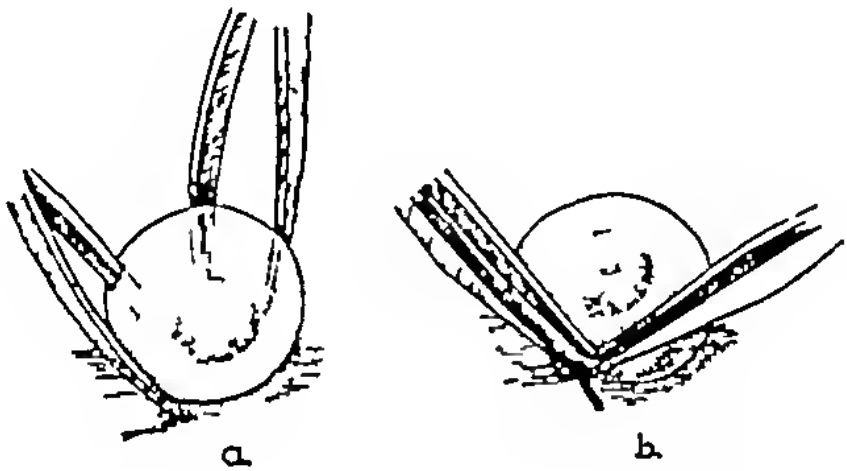

FIG. 3 —Removal of the jelly membranes of an *Ambystoma* egg (see text)

Ambystoma species a thin sticky layer forms the outermost covering In *A maculatum* and *tigrinum* the eggs are imbedded in a common jelly, which probably originates by coalescence of individual soft jelly layers. The eggs of *A opacum* are laid singly under leaves, etc. (see p 16) Their outermost sticky membrane is usually covered with mud particles.

Two pairs of watchmaker forceps with carefully sharpened points are needed for the removal of the membranes. The eggs of *A maculatum* and *A tigrinum* are first taken out of their common jelly mass those of *A opacum* are placed in water and allowed to swell to capacity The eggs are transferred into a Petri dish or other glass dish with no Permoplast or agar ground Perform all further manipulations under the low power binocular dissection microscope The two outermost layers—the sticky thin membrane and the outer capsule—can be easily removed together Next follows the inner capsule which is under the pressure of the capsular fluid Remove it in the following way (Fig 3) Set the forceps in your

right hand firmly on the glass bottom with extended prongs and push the egg against it Pierce the capsule with one prong of the left forceps (Fig 3 *a*) Carefully avoid injury to the egg No fluid will escape because the forceps plug the hole Next close the left forceps and hold a firm grip on the capsular membrane Next insert one point of the right forceps into the hole alongside the left forceps (Fig 3 *b*) and rupture the membrane with a quick jerk of both pairs of forceps in opposite directions The capsular fluid will then escape and the egg will pop out or it can be shaken out. The removal of the vitelline membrane is rather difficult in young stages For this purpose the forceps must be sharpened to the finest points possible The removal is usually unsuccessful in cleavage stages. In blastula and gastrula stages the vitelline membrane is still very closely applied to the egg but can be removed in the following way Grasp the vitelline membrane with the left forceps at the animal pole or over the blastopore and tear a large hole in it with the right forceps If you do not succeed then puncture the egg at two points on the animal pole with a glass needle or the point of the forceps The perivitelline fluid will escape and the egg will collapse slightly wrinkles will appear on the egg surface and make it possible to grasp and rupture the membrane After removal of all membranes the egg will collapse It is very delicate and must be handled with extreme care If used for operations it should be washed in several changes of sterile Holtfreter solution and placed in a dish with agar bottom in $\frac{1}{5}$ Holtfreter solution If the egg was punctured place it in full Holtfreter solution for a while to facilitate healing Use sterilized pipettes and dishes and keep all dishes covered

Triturus —In the eggs of *Triturus* the outer capsule including the outermost sticky membrane, can be removed with little difficulty However the pressure of the capsular fluid is much greater than in *Ambystoma* eggs and the removal of the inner capsule is therefore more difficult. To recover the embryo without injury it is necessary to tear a large hole in the capsule at the first attempt Pull the forceps apart with a rapid jerk. The tension of the capsular fluid is low for a short period immediately after fertilization and workers who use *Triturus* eggs in cleavage and gastrula stages sometimes decapsulate them shortly after laying During cleavage and up to tail bud stages it is very difficult to remove the capsule successfully For this reason *Triturus* eggs are not recommended for use in classroom experiments in stages earlier than tail bud stages

Frogs —Frog eggs are imbedded in a common jelly mass and individually surrounded by loose jelly layers which however form no tough elastic capsules The jelly is not difficult to remove One may cut it off with forceps or roll the eggs on filter paper It is difficult to remove the

vitelline membrane in early stages up to neurulae Besides decapsulated anuran eggs are very soft and extremely delicate. They collapse even more than *Triturus* eggs Frog gastrulae are therefore not suitable for classroom experiments but, in embryos from late neurula stages on, all membranes, including the vitelline membrane can be easily removed

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d) NARCOSIS

Amphibians in tail bud stages are ciliated and rotate within the capsular fluid The ciliary beat is strong enough to keep the animal in slow motion when it is taken out of the membranes This may be an impediment in operations The only way to hold the embryo tight is to bury it in a Permoplast groove or under glass bridges

The first muscular motility begins in stages corresponding to H31 or 32 for *A maculatum* (see p 125) From these stages on, the embryos must be narcotized for operations and for protocoling Two excellent narcotics are at our disposal neither of which has a detrimental effect on the embryo if applied in proper dosage.

1 *Chloretone* (*acetone chloroform*) —It will dissolve rather sparingly in any culture medium Shake thoroughly Keep bottles tightly stoppered and dishes covered In most cases a concentration of 1 3 000 will be satisfactory Old larvae may require a stronger concentration It is advisable to try out the effectiveness of the concentration before valuable material is narcotized Embryos and larvae become immobile within a few minutes and recover within 5–10 minutes The heart beat should be watched Its stoppage is a sign of too high concentration embryos can be saved if they are transferred immediately to a normal medium Embryos may be kept under light narcotization for several days (see p 127 also Matthews and Detwiler 1926)

2 *MS 222* [4]—This is a methan sulfonate of meta amino-benzoic-acid ethyl-ester an isomer of anesthesin (Rothlin 1932) It is soluble in water and even less toxic than chloretone The animals recover more rapidly than from chloretone Copenhaver (1939) finds that the heart beat is only slightly affected Concentrations of 1 2 000 or 1 3 000 are recom

[4] MS 222 may be obtained from Sandoz Chemical Works, Inc., 68–70 Charlton St., New York City

mended Schotté and Butler (1941) report that 'a stock solution of 1 1000 can be sterilized in the autoclave without apparent loss of narcotic properties and with no increase in toxicity Again, a normal heart beat is the best indicator for a proper dosis Embryos can be kept under light MS anesthesia for several days

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e) STANDARD EQUIPMENT FOR OPERATIONS ON AMPHIBIAN EMBRYOS[5]

1 EQUIPMENT FOR EACH STUDENT

Dishes

several 4 inch finger bowls or No 4 Lily cups to keep eggs or egg masses before operation

several Petri dishes or Syracuse dishes for decapsulation (p 37)

several Syracuse dishes with Permoplast ground (operation dishes)

6–12 Syracuse dishes section dishes or small paraffined Lily cups for raising of operated embryos

1 glass jar laid out with cotton for alcohol sterilization of metal instruments

Instruments

binocular low power dissecting *microscope*

microscope lamp

2 pairs of watchmaker forceps

1 pair of ordinary small forceps

several glass needles

several hair loops

2–3 glass rods with ball tips of different sizes

several glass bridges of different sizes

2 medicine droppers

2 wide mouthed pipettes (about 5 mm in diameter to transfer whole eggs)

wooden holder for glass instruments

[5] See Part I (p 3) for all details.

Dishes

several crystallizing dishes or 7½ inch finger bowls for egg masses

Other equipment

materials for preparing of glass instruments (see p 3)

Permoplast

oil stone for sharpening of watchmaker forceps

Solutions

full strength Holtfreter solution (for operations)

¼ or $\frac{1}{10}$ Holtfreter solution (for rearing of embryos)

MS 222, 1 3 000 or chloretone 1 3,000 (narcotics)

70 per cent alcohol for sterilization of metal instruments

Note Holtfreter solutions must be sterilized for operations on early stages (gastrulae)

f) SOME GENERAL RULES FOR OPERATIONS

Prepare a sufficient number of glass instruments, particularly glass needles, to have them on hand if an instrument should break during the operation

Use only the best living material for operations. Discard all embryos which do not gastrulate properly, which lose individual cells on the surface which show signs of edema, which show irregular pigmentation or other signs of an unhealthy condition

Sterilize and clean all instruments carefully Keep the operating table clean Wash the embryos after removal of the jelly membranes in sterile Holtfreter solution Keep all dishes covered Remove dead animals at once Mark all pipettes and instruments used for fixation with a label never use them for living material and keep them off the operating table

Choose the proper optical magnification Operate under medium rather than highest power Be sure to have an optimal illumination of the visual field

Operate in full strength Holtfreter solution After healing is well under way transfer the embryos to ¼ or $\frac{1}{10}$ Holtfreter solution

Always make several operations of the same type The mortality in operations on embryos is always high despite skill and cleanliness

Keep operated embryos in a cool place to hold back bacterial growth etc Carefully shield off direct sunlight

Fix operated embryos when they show signs of disintegration This process once started goes on rapidly and the embryo may be completely disintegrated a few hours later

Consider protocols and observations equally as important as the operations. Get a clear understanding of the theoretical implications of every experiment which you perform and try to evaluate in your own words your results in conjunction with those of the class

2. THE PROSPECTIVE SIGNIFICANCE OF EMBRYONIC AREAS (VITAL STAINING)

a) A DESCRIPTION OF GASTRULATION IN *Urodela*

An intimate knowledge of the process of gastrulation in amphibians is indispensable for an understanding of the experimental work done on early embryos to be described in the following pages During gastrulation the germ layers are formed by extensive cell movements. Moreover, transplantation explantation and other experiments have shown that, during and shortly after gastrulation, some of the main organ primordia become more or less rigidly 'determined. In other words profound changes in the visible and invisible organization of the embryo take place during this period The morphogenetic cell movements are now clearly understood thanks to the admirable vital staining experiments of Walther Vogt (1925 1929) and his collaborators The following presentation is largely based on their work.

1 THE STRUCTURE OF THE URODELE EMBRYO BEFORE AND AFTER GASTRULATION

The blastula is spherical and clearly polarized The animal hemisphere is characterized by several layers of small cells which form the thin roof of the 'blastocoele. They contain little yolk and are usually pigmented The cells of the "vegetal hemisphere are heavily laden with yolk and are unpigmented

At the end of gastrulation i e shortly before the medullary plate makes its appearance the embryo (Figs 9 10) is still spherical in shape but it has acquired a bilateral symmetry and its walls are formed by three sheets the germ layers The blastocoele is almost entirely replaced by another central cavity—the primitive gut or archenteron The archenteron is actually closed and plugged by the yolk plug up to early neurula stages It opens to the outside by withdrawal of the plug (Brown 1941) The ectoderm forms a complete outer covering continuous with the mesoderm around the blastopore It is usually pigmented throughout the unpigmented cells have been shifted inside The mesoderm forms a mantle subjacent to the ectoderm However the anterior ventral part of

the embryo remains free of mesoderm, and the mesoderm mantle ends with a free edge, which extends in an oblique direction from dorsal-anterior to ventral posterior (*me* in Fig 5) This sharp edge fades out near the mid-dorsal line, where the anterior-dorsal part of the mesoderm mantle merges with the anterior-dorsal part of the entoderm The anterior part of the archenteron is the foregut, which is disproportionately wide at this stage The entoderm behind the foregut forms a troughlike structure partly inside of and covered by, the mesoderm mantle. Its massive floor is formed by the large yolk laden cells Its anterior and lateral walls are thinner and rise steeply from the floor The walls do not meet in the mid dorsal line (at least not in the stage under consideration) but appear as two parallel lines, lateral to the median plane (*e*, in Figs. 7–10) Thus the archenteron has a dorsal gap and no entodermal roof However the dorsal part of the medoserm mantle becomes intimately applied to the free edges of the entodermal trough and thus forms temporarily a lid over the archenteron for this reason the dorsal part of the mesoderm mantle at this stage has been given the misleading name "archenteron roof In the neurula stage the free entoderm edges will converge and eventually, fuse underneath the mesodermal mantle thus giving the archenteron its permanent entodermal roof The temporary contact of archenteron roof and entoderm is so intimate that cross-sections may simulate an actual fusion Such pictures were taken as evidence in favor of the entodermal origin of the mesoderm and particularly of the notochord The investigations of Vogt and of others before him leave no doubt that this conception is erroneous, both germ layers originate at the blastopore and remain separate units, despite their temporary contact

This latter statement requires a qualification There is, indeed, true continuity of mesoderm and entoderm at two places in a narrow sickle shaped area ventral to the blastopore and as mentioned before in the roof of the headgut. The situation in this latter area is difficult to visualize and deserves further comment. *Ch* in Figure 9 marks the anterior end of the prospective notochord At this point the mesoderm does not end abruptly as will the notochord in later stages, but continues into the so-called ' prechordal plate In its cellular texture the prechordal plate appears as a true transitional zone between ento- and mesodermal structures A sagittal section exactly through the median plane shows therefore a continuous archenteron roof partly of entodermal and partly of mesodermal origin Such sections figure prominently in most textbooks. Yet they are liable to give a wrong conception of the entoderm mesoderm relation unless they are presented in conjunction with transverse sections

The transformation of the blastula into the gastrula is accomplished by a sequence of integrated cell movements The greater part of the vegetal hemisphere invaginates into the interior around the blastopore The animal hemisphere spreads and overgrows the vegetal hemisphere and eventually forms the entire surface of the neurula During this process the blastopore changes its shape continuously These changes vary in different forms and will be described for *A maculatum* In Harrison's stage series only 3 gastrulation stages (H10–H12) are distinguished This proved to be insufficient for experimental workers Lehmann (1926) and Boell and Needham (1939) have inserted several intermediate stages We have adopted the seriation of the latter authors and have added another intermediate stage (H12½) We distinguish the following stages (Fig 45)

Stage	
H9	blastula
H10	early blastopore
H10½	sickle-shaped blastopore
H10¾	semicircular blastopore (¾ moon)
H11	horseshoe-shaped blastopore (¾ moon)
H11¼	large yolk-plug stage
H12	small yolk plug stage
H12½	slit-shaped blastopore
H13	neural groove stage

The incipient blastopore appears as an irregular line between the equator and the vegetal pole (stage H10) It assumes the shape of a sickle (stage H10½) and acquires a marked bilateral symmetry Its plane of symmetry which coincides with that of the future embryo only now becomes apparent although it is determined much earlier[1] The region of the animal pole is the future anterior end of the embryo the blastopore itself marks the posterior end The line connecting the animal pole with the blastopore is the future mid-dorsal line The area above the blastopore is the so-called upper or dorsal lip of the blastopore Next the blastopore begins to elongate and to encircle the yolk field When it is semicircular (stage H10¾) the areas lateral to it begin to invaginate around the lateral lips The blastopore then assumes the shape of a horseshoe (stage H11) Eventually the lateral invagination grooves complete the encirclement of the yolk field which gradually disappears to the inside (formation of a ventral lip) The exposed part of the yolk in the stage of the circular blastopore is called yolk plug (stage H11¼) The rapid in

1 some species a gray crescent appears shortly after fertilization in the region of the future upper lip of the blastopore and its plane of symmetry already marks that of the embryo

ward movement of the yolk continues, the yolk plug becomes small (stage H12) and eventually disappears entirely The blastopore has now assumed the shape of a short slit (stage H12½), which extends in longitudinal direction (i.e , perpendicular to the early blastopore) and marks the future anus This stage is conventionally considered the end of gastrulation. Shortly afterward the medullary plate becomes visible (stage H13) From this stage on up to that of the closed neural tube, the embryo is called a neurula

3 W VOGT'S METHOD OF LOCALIZED VITAL STAINING AND ITS GENERAL RESULTS

Gastrulation is primarily a phenomenon of cell movements and not of cell division and proliferation Many attempts have been made to study the cell movements, e g , by inserting a fine glass needle into the blastula wall and following its shift. This and similar methods are inadequate for several reasons The most serious objection is that such mechanical devices may interfere with normal development. Decisive progress was made when W Vogt applied a method of localized vital staining[1] The procedure is briefly, as follows A small particle of agar stained with Nile blue sulphate or neutral red is pressed against the surface of the blastula for a short period Such marks remain distinct and well circumscribed for several days According to Vogt diffusion of the stain into neighboring cell areas is negligible and the marks do not interfere with normal development. In this way it is possible to follow the movements of the marks throughout gastrulation by continuous observation Particularly instructive were those experiments in which blastulae or early gastrulae were marked by a series of alternating red and blue marks (as many as fourteen on one embryo) and their shifts relative to each other observed In an exhaustive analysis Vogt obtained almost complete records of the gastrulation movements of all parts of the surface area, and eventually he was in a position to outline a coherent picture of the mechanics of germ layer formation The same experiments solved another problem of no less importance Since the marks persisted over a considerable time it was possible to establish their ultimate locations in the organ primordia by microdissection of early tail bud stages. This in turn, enabled Vogt to project the pattern of the organs back onto the surface of the blastula or of the early gastrula. His maps of the organ forming areas of blastulae and early gastrulae are well known. They are an invaluable help not only for a better understanding of gastrulation but as guides in transplantation and other experiments

[1] Similar methods had been devised previously by Goodale (1911) and Detwiler (1917)

The limitations of Vogt s method should be clearly understood. The designations of the different areas on the maps indicate merely their ac tual fate ("prospective significance Driesch) in normal, undisturbed de velopment They do not imply that the early gastrula is built up of dis-crete mosaic stones which differ actually from one another in structure or otherwise Such a view would misinterpret entirely the methodological rank of the vital staining technique It is a tool for refined observation of normal development i.e. a descriptive method not an analytical method It does not reveal intrinsic properties or potencies of the stained areas Only potency tests like transplantation or isolation experiments are suit able for such an analysis It is correct to refer to the areas on the map as prospective notochord etc but not as notochord or notochord primordium

4 MAPS OF THE EARLY URODELE GASTRULA

Maps of the early gastrula are reproduced in Figure 4 Similar maps for the urodele blastula and for the anuran gastrula may be found in Vogt (1929) and Pasteels (1942) The map requires little comment Its out standing landmark is the line (*sl*) which separates the invaginating ma terial (prospective entoderm and mesoderm) from the noninvaginating prospective ectoderm The prospective mesoderm forms a ring or girdle around the yolk field. It is broadest on the dorsal side and narrowest on the ventral side In the German literature it is known as the *Randzone* (marginal zone) The most peculiar feature of the map is that the areas which will form the axial organs have their greatest extent in a di rection perpendicular to the median plane i.e perpendicular to their ulti mate position Thus the prospective medullary plate area forms a trans-verse band with pointed lateral ends the prospective notochord is a sickle shaped area above the blastopore the somites are lined up in two transverse rows etc. It requires a considerable wheeling to maneuver all prospective areas into their ultimate positions

Vogt s map which was largely based on experiments on the European *Triturus* species has been revised by Nakamura (1938) using the Japa nese newt, *Tr pyrrhogaster* and by Pasteels (1942) using the axolotl (*A mexicanum*) The maps of Pasteels are reproduced in Figure 4 *C* and *D* they apply probably to other *Ambystoma* species as well Both authors are in virtual agreement with each other Their maps are in all essential points identical with those of Vogt but differ from the latter in several de-tails as follows

The shape of the prospective notochord area is different in particular its lateral borns are less pointed and do not extend as far lateral as they

do in Vogt s map According to Pasteels, the ventral marginal zone is large ly prospective lateral plate material (*l*), whereas Vogt considers the greater part of this area as prospective trunk and tail somite material (*t*) Accord ing to Pasteels the latter material extends to the median dorsal line and

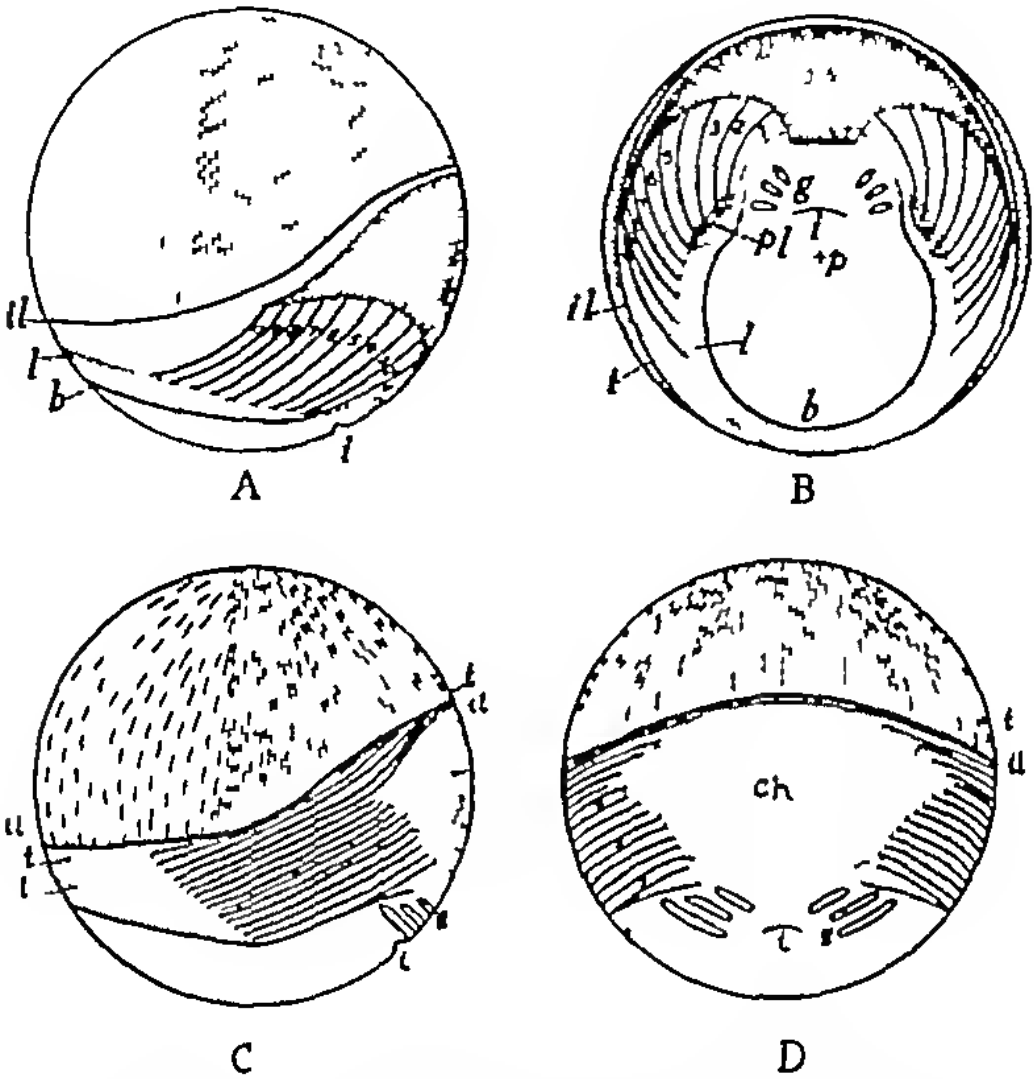

FIG 4 —Map of prospective areas of urodele embryos at the beginning of gastrulation. *A* lateral view *B* dorsal view of *Triturus* (from Child 1941 after Vogt) *C* lateral view *D* dorsal iew of axolotl (after Pasteels, 1942) Denser broken lines, neural plate less dense broken lines, general ectoderm course stippling notochord fine stippling mesoderm *b* ventral lip of blastopore *ch* notochord *g* gill area *l* beginning of invagination *ll* limit of invagination *l* lateral mesoderm *p* vegetal pole *pl* pronephros and forelimb area *t*, prospective tail region 1–10 somites 1–10

forms a narrow strip between the prospective notochord and medullary plate Furthermore Pasteels has made detailed studies of the origin of the different parts of the somites which on his map form very long and narrow strips As was to be expected that part of the prospective somite region which is adjacent to the prospective notochord material represents

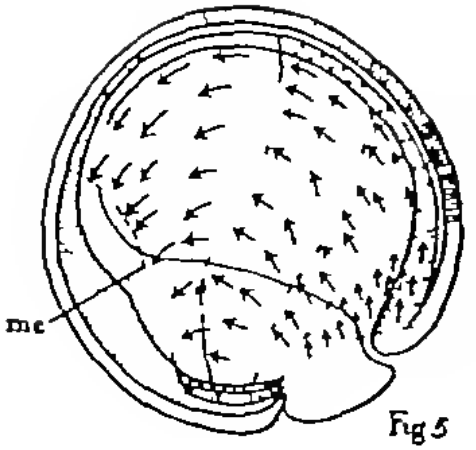

Fig 5

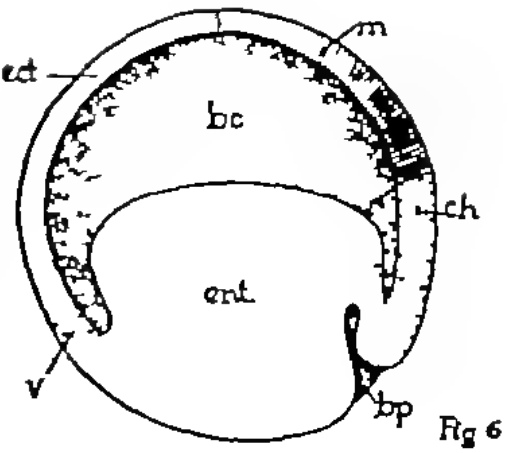

Fig 6

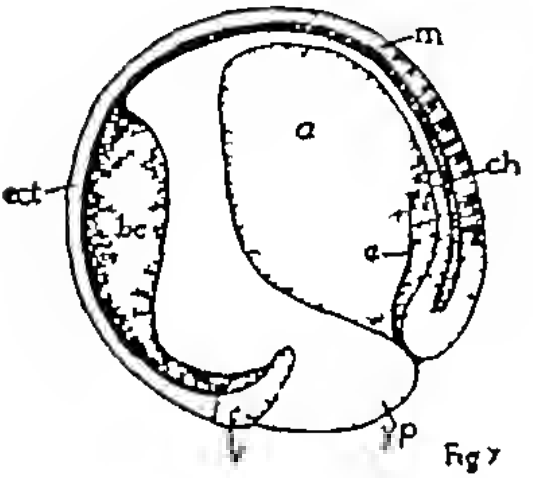

Fig 7

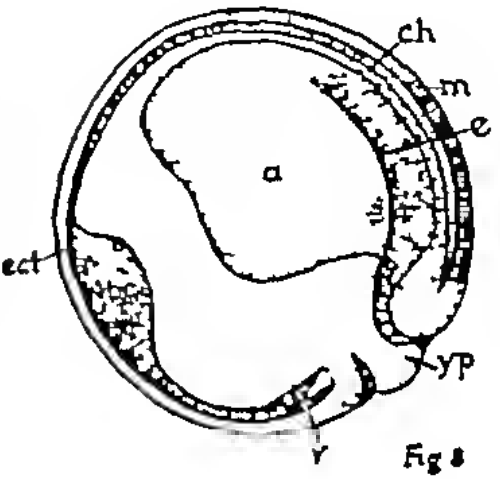

Fig 8

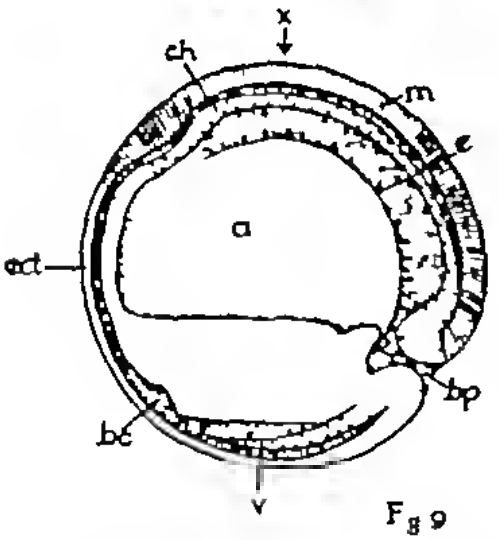

Fig 9

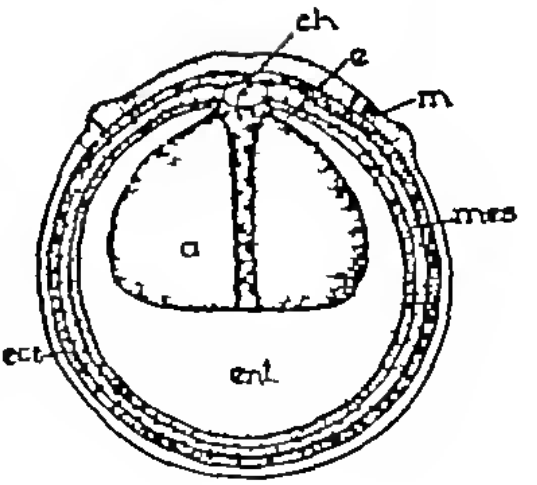

Fig 10

Fig 5 —Reconstruction of the movements of the mesoderm mantle, projected on a middle gastrula stage (urodele) The dotted lines indicate the anterior edge of the mantle in four different stages *me* = anterior mesoderm border at the end of gastrulation (Fig. 9) The area anterior to *me* is the "mesoderm free field" The arrows indicate the directions of movements (after Vogt, 1929)

Figs 6–10 —Gastrulation in urodeles (reconstruction after diagrams and sections, in Vogt, 1929) Fig 6 beginning of gastrulation. Fig 7 large yolk-plug stage Fig 8, small yolk-plug stage Figs 9–10 early medullary-plate stage Figs. 6–9, median sections Fig 10, transverse section cut in plane *x* of Fig 9 posterior half of neurula. *a* = archenteron *bc* = blastocoele *bp* = blastopore *ch* = notochord *e* = upper edge of the entoderm trough, *ect* = ectoderm, *ent* = entoderm *m* = medullary plate material *v* = ventral mesoderm, *yp* = yolk plug.

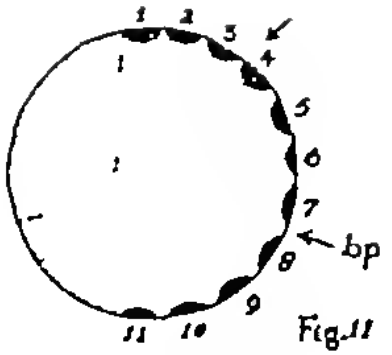

Fig. 11

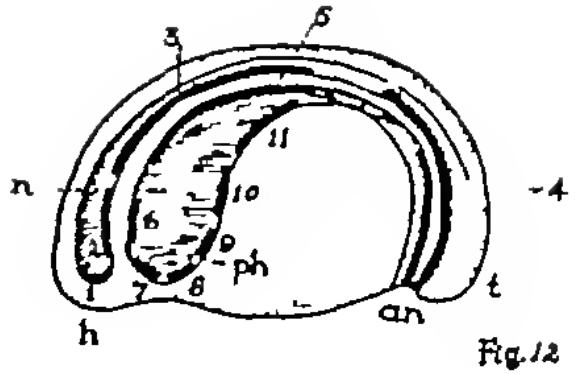

Fig. 12

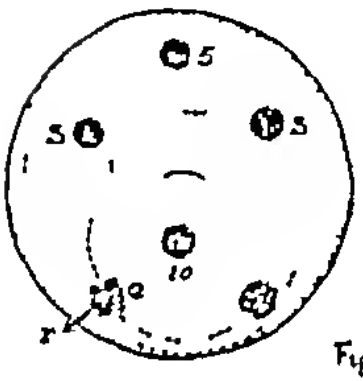

Fig 13

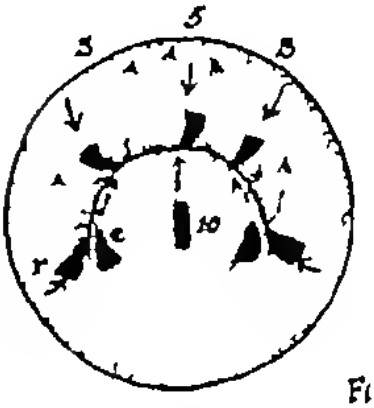

Fig 14

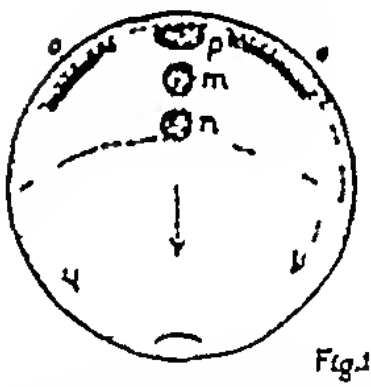

Fig. 15

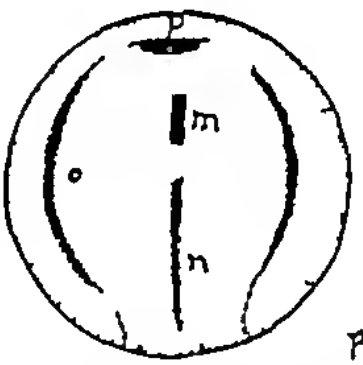

Fig 16

FIGS 11–16 —Vital-staining experiments on urodele gastrulae (Figs 11–14 after Vogt 1929 Figs 15 16 after Goerttler 1925)

FIG 11 —Eleven marks (*1–11*) placed in the median line of a late blastula. Lateral view The upper arrow indicates the border of invagination *bp* = point of origin of blastopore. The dotted lines indicate the borders between the main prospective areas (see Fig 4 *A*)

FIG 12 —The same embryo in early tail-bud stage, with position of marks *1 11* *An* = anus *h* = head *n* = neural tube *ph* = pharynx *t* = tail.

FIG 13 —Marking of prospective mesoderm and entoderm in the early gastrula. Ventral view The dotted lines indicate the borders between prospective areas (see Fig 4, *B*) Marks *5* (prospective notochord) and *10* (median entoderm) as in Fig 11; *e* = mark on outer edge of entoderm field = mark on ventrolateral part of marginal zone (prospective lateral mesoderm) *s* = mark on anterior somite material

FIG 14 —The same embryo as in Fig 13 in middle gastrula stage Note the changes in shape and in position of the marks. The solid arrows indicate the direction of past movements on the surface the dotted arrows indicate the movements after Invagination Designations as in Fig 13

FIG 15 —Marking in the prospective medullary plate area (dotted lines) of the early gastrula stage *m n* = marks in the median line *e* = lateral marks *p* = mark on the animal pole The arrows indicate the directions of movements

FIG 6 —The same embryo as in Fig. 15 in medullary plate stage.

the inner median edges of the future somites and the part adjacent to the prospective lateral plate area (*l*) represents the ventral edges of the somites The outer lateral borders of the somites cannot be stained by superficial marks and must therefore be located in the deeper layers of the marginal zone (see p 53)

5 THE FORMATION OF THE MESODERM

The mesoderm mantle is formed by an invagination of the mesoderm girdle or marginal zone around the blastopore *Invagination* is one of the four basic gastrulation movements as distinguished by Vogt. The fact that the blastopore makes its first appearance in a dorsal position and gradually encircles the yolk indicates a definite sequence in time of the invagination of different mesoderm areas. The dorsal mesoderm (prospective notochord) invaginates first somite material follows around the lateral lips eventually the ventral part of the marginal zone is tucked in As a general rule areas which are located nearest to the blastopore will invaginate first and their final position inside will be farthest away from the blastopore material which invaginates late will settle near the blastopore

The map shows that the blastopore originates entirely within the yolk field Therefore prospective entoderm will be the first material to invaginate it will be carried into the head region and will form there the anterior blind end of the archenteron that is the anterior parts of floor walls and roof of the future pharynx. Accordingly, the gill slits are mapped out on the early gastrula at a short distance from the early blastopore The dorsal entoderm is immediately followed by the prechordal material which in turn is followed by the anterior end of the prospective notochord material This then is the first mesodermal area to be tucked under In order to visualize clearly the fashion in which the notochord material is brought into its final position let us follow two marks placed on prospective notochord areas Mark 5 (Figs 11 13) is located in the median plane and has a central position in the notochord area It will move toward the blastopore invaginate around its dorsal lip and disappear While it is still outside it will change its shape it will elongate and at the same time become slightly narrower this expansion in the longitudinal direction will continue after its invagination Eventually the formerly circular mark will stain a surprisingly long strip of the narrow notochord Mark 5 (Fig 12) illustrates well the enormous degree of elongation which the prospective notochordal material undergoes Almost all parts of the gastrula undergo varying degrees of expansion during gastrulation *Elongation or expansion* is the second basic gastrulation movement

placed in the median line immediately above and below the blastopore il lustrate the situation The lumen of the head gut swells rapidly and exten sively in the first phases of gastrulation whereby the lumen of the blasto-coele becomes obliterated While the notochord and somite materials follow the head-gut material around the dorsal and lateral lips in true in vagination movements, the ventral yolk glides into the interior under neath the arch of the sickle and horseshoe shaped blastopore as a continu ous stream without actual invagination around a groove, a ventral lip is nonexistent during these phases of gastrulation Vogt once compared this shift with the retraction of a stretched-out tongue. A mark placed in the middle of the yolk field (10 in Figs. 13 and 14) illustrates this movement. The mark elongates while it approaches the blastopore and disappears un der the blastoporal groove Having arrived inside it moves forward and will be found eventually as a broad patch in the middle of the floor of the intestine (Fig 12) It can be shown that all prospective entoderm material which was located in the median line before invagination will form the median floor of the intestine Obviously lateral parts of the yolk field will form the lateral walls of the archenteron trough. Mark *e* in Figure 13 likewise moves toward the blastopore and elongates in a direction almost parallel to mark 10 In its progression inside, it spreads farther and at the same time, moves upward and converges toward the median plane. It will be found eventually in the upper edge of the left wall of the archen-teron. A mark between 10 and *e* would stain an area in the middle of the lateral wall of the archenteron Again, regions near the blastopore will form anterior parts of the intestine, and regions at a distance from the blastopore will invaginate later and form posterior intestine. The ento-derm formation is completed with the disappearance of the yolk plug (Fig 9) and no "late invagination of entoderm occurs

A new problem arises when we visualize mesodermal marginal zone and entodermal yolk field as being continuous on the surface of the blastula but entirely separate structures at the end of gastrulation (except in the pharyngeal and in the blastoporal region) Even their directions of move ment inside are divergent The mesoderm mantle spreads forward and downward the walls of the entoderm move upward Their separation must occur sometime during gastrulation According to an earlier view, which was widely accepted for a long time this would happen by invagi nation of a uniform archenteron and subsequent delamination of the mesoderm from the entoderm The mesoderm would be a derivative of the entoderm i e of gastral origin According to an alternative inter pretation the separation takes place before or during invagination and the two germ layers invaginate as autonomous units peristomial on

gin of the mesoderm It is one of the outstanding contributions of Vogt to a theory of gastrulation to have established for *Urodela* the correctness of the second alternative. If one considers for a moment marks *e* and *r* (Fig 13) as one single mark, then one finds that this mark is cut in two at the moment when it arrives at the blastoporal groove (Fig 14) From then on the two parts take entirely different courses (see arrows) and eventually are widely separated—one in the lateral plate the other at the upper edge of the entodermal trough Marks which are partly on entodermal and partly on mesodermal territory were actually studied and the reality of the rupture was demonstrated beyond doubt. Accordingly the horseshoe shaped line on the map (heavy in Fig 4, *B* and stippled in Fig 13) designates more than the border line between entoderm and mesoderm it demarcates the line of rupture and its absence (on the map) between prospective gills and first somites merely expresses the fact that entoderm and mesoderm will remain continuous in the pharyngeal region

7 THE GASTRULATION MOVEMENTS OF THE PROSPECTIVE ECTODERM

The gastrulation movements of the prospective ectoderm (Figs 15 and 16) were studied by Vogt s collaborator K. Goerttler (1925) and by Schechtman (1932 for *Tr torosus*) Since the embryo retains its size and its spherical shape throughout gastrulation the animal hemisphere (prospective ectoderm) must be expected to compensate for the invaginating ventral hemisphere by extensive expansion and thinning This is demonstrated by every mark placed on the prospective ectoderm except on the animal pole The extent and direction of the movements of different parts of the ectoderm will be discussed separately for prospective epidermis and prospective medullary plate

Prospective medullary plate—If the animal pole is stained (Fig 15 *p*) then the mark will be found first, in the anterior transverse part of the medullary fold and later on in the floor of the forebrain Its shape is almost unaltered The animal pole then is the only area of the gastrula which remains stationary Marks placed in the median line will stay in the midline and elongate in the direction toward the blastopore The nearer to the blastopore i.e the nearer to the future posterior end the more will a mark elongate during gastrulation and neurulation (compare *m* and *n*) The median marks will be found in the floor of the spinal cord It is important to notice that all material which is located in the median line of the early gastrula remains there and thus makes true concrescence (i e growing together) of lateral areas impossible The lateral parts converge toward the median line but never concresce The same was stated before for the notochord (True concrescence takes place when

the neural folds fuse or in heart development, but nowhere in gastrulation) Lateral marks show clearly the convergence of the lateral parts of the prospective medullary material Mark *o* in Figure 15 is particularly suitable to illustrate the "wheeling movement (*Schwenkung*, Goerttler) toward the median line the fixed point being the median end of the mark near the animal pole The parts of the mark which are farthest away from the midline traverse the longest distance This wheeling movement takes place largely during the first part of gastrulation it is followed, during the later phases of gastrulation by elongation

The prospective *epidermis* occupies the ventral sector of the animal hemisphere Its movements are in conformity with those of the prospective medullary material They are characterized by a very considerable expansion in a fanlike fashion In ventral view this expansion appears as a "divergence The old term epiboly' ("growing over') may well be applied to this maneuver, since this spreading is at the same time, a process of growing over the invaginating mesoderm and entoderm.

8. SUMMARY

The prospective medullary area and the prospective notochord have several features in common Their longest diameter is in a transverse direction before gastrulation and in longitudinal direction afterward The gastrulation movements of their median as well as of their lateral parts are almost identical although the one invaginates and the other does not. Their movements are perfectly integrated with each other, since they have a long border in common along which they remain continuous throughout gastulation A similar comparison may be drawn between the divergence of ventral and ventrolateral ectoderm and that of ventral and ventrolateral mesoderm All these observations taken together illustrate emphatically the integration of all gastrulation movements the uniformity of the process as a whole, whose basic trends—elongation convergence divergence etc.—transcend the border lines of invaginating and noninvaginating areas and of the prospective germ layers

b) PREPARATION OF DYED AGAR

Nile blue sulphate and neutral red are generally used as vital (non toxic) dyes Prepare a 1–2 per cent solution of agar (c.p , powder or shreds) in distilled water Boil briefly Pour thin films of the solution while it is still warm on carefully cleaned microscope slides or on larger glass plates Allow them to dry thoroughly (1 or more days) Place the agar plates in a large volume of 1 per cent Nile blue sulphate or 1 per cent neutral red and let them stand for 1 or more days Wash off the excessive dye and allow the agar to dry again The plate can be kept indefinitely

in a dustproof wrapping Before use, moisten a small area with a drop of water After the agar is swollen (1–2 min) scrape off narrow strips of the agar film with a scalpel. Dyed agar plates may also be kept in 70 per cent alcohol, which must be rinsed off very carefully before use

c) VITAL STAINING EXPERIMENTS ON THE EARLY GASTRULA OF *Urodela*

Read carefully section 2a consult Vogt s and Pasteels maps of the prospective regions of the urodele embryo (Fig 4)

Material for Experiments 1–4

Ambystoma opacum punctatum or *tigrinum*
Triturus pyrrhogaster or *Tr torosus*
stage H10 or H10½ (p 45)
agar stained with neutral red
standard equipment (p 41)

EXPERIMENT 1 STAINING OF THE UPPER LIP OF THE BLASTOPORE (PROSPECTIVE NOTOCHORD)

Procedure

Select a number of healthy gastrulae Remove the outer jelly membranes but leave the vitelline membrane intact. Wash the embryos in sterile $\frac{1}{10}$ Holtfreter solution and transfer them to an operation dish With the glass ball make a depression in the Permoplast into which a gastrula will fit tightly Transfer a glass bridge of suitable size and a piece of red or blue agar to the operation dish Under the binocular microscope cut out a square piece of agar which will cover the median one third of the upper lip area Place the embryo in the depression The embryo will usually rotate into a position in which the blastopore is not visible Sometimes one succeeds in moving the embryo back with the hair loop If this does not work apply one of the following techniques Turn the blastopore upward and while the embryo rotates slowly back to its former position mark the position of the blastopore on the Permoplast at the edge of the groove After the embryo has come to rest, push a piece of stained agar between the Permoplast and the embryo at the marked point Press the embryo against the agar by gentle pressure with a glass bridge Another trick is to puncture the vitelline membrane (and the embryo) in several places with a very fine glass needle This will partly release the inner pressure and the embryo will remain in a position with the blastopore upward The fine holes will heal at once Place the agar on the median part of the upper lip (5 in Fig 13) and press it against the embryo with a glass bridge Stain for 15–45 minutes (depending on how deeply stained the

agar is) Remove the glass bridge and the agar very cautiously with a hair loop and transfer the embryo to a dish with $\frac{1}{4}$ Holtfreter solution.

Protocols —Protocols are of prime importance Label the embryo and make a sketch indicating the position of the mark. Observe the embryo once or twice daily and make a series of sketches Observe and record in the sketches the changes in position and in shape of the mark. Note its invagination and elongation After invagination a deep stain may be visible through the epidermis

Dissection —Dissect the embryo after it has reached the tail-bud stage, either alive or immediately after a short fixation in 10 per cent formaldehyde (the dye will fade out slowly in formaldehyde) Mount it in a Permoplast depression with its dorsal side upward Very cautiously remove the dorsal epidermis, the brain, and spinal cord using the glass needle and hair loop thus exposing the notochord and somites Make a sketch, indicating precisely the position and extent of the stained area In the protocol state your results clearly

EXPERIMENT 2 VITAL STAINING OF THE PROSPECTIVE SOMITES

Stain the left or right one third of the upper blastopore area (5 in Fig 13) Proceed in all details as before Observe the invagination and dissect the embryo in an early tail bud stage

EXPERIMENT 3 VITAL STAINING OF THE PROSPECTIVE MEDULLARY PLATE

Again mark on the Permoplast the position of the blastopore or puncture the embryo Place a mark between the blastopore and the animal pole nearer to the latter During the following days note the elongation of the stained material If the mark was not exactly in the median plane note the convergence of the mark toward the median plane, in addition to its elongation (see Figs 15–16)

EXPERIMENT 4 VITAL STAINING OF THE ANIMAL POLE

Note that the mark scarcely changes its position and shape It will be found in the anterior part of the head (*p* in Figs 15 and 16)

d) VITAL STAINING OF AREAS OF THE NEURULA OF *Urodela*

Material for Experiments 5–7

Ambystoma any species
stages H14 and H15
agar stained with neutral red
standard equipment (p 41)

Experiment 5 Vital Staining of the Prospective Eye forming Area

(Manchot 1929)

Procedure

Remove all membranes including the vitelline membrane Place a mark on the anterior median part of the medullary plate The mark should cover the median one-third or one fourth of the plate Its anterior border should cover the slope of the transverse medullary fold, in stage H15 (Fig 17, *a*) Allow the embryo to develop to stage H26 or stage H29

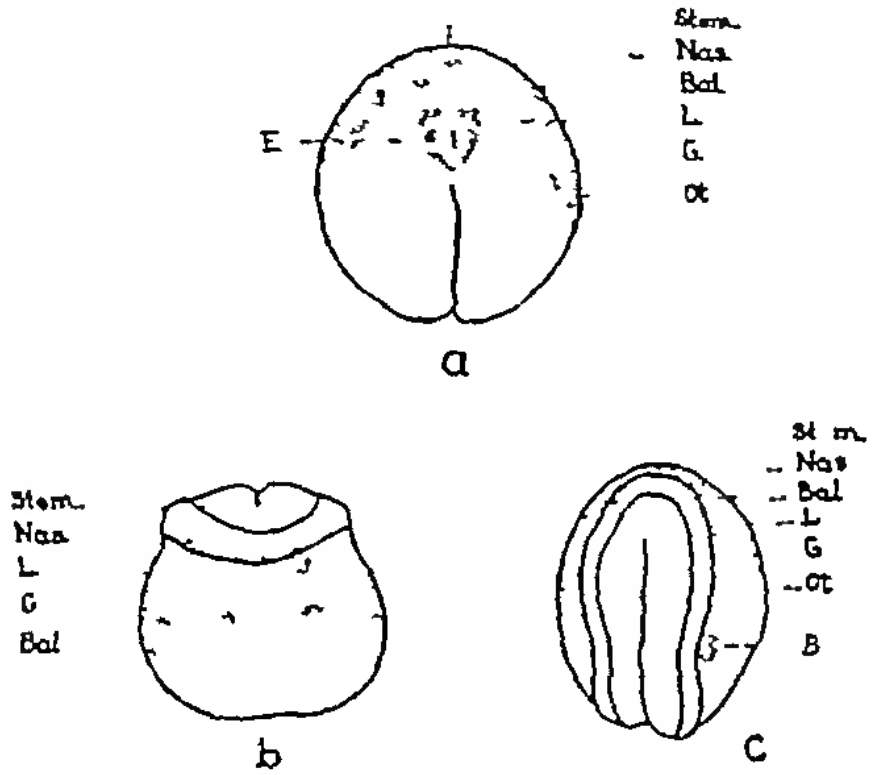

Fig. 17 —Vital staining of head structures (after Carpenter 1937) *B* = border between head and trunk, *Bal* = balancer *E* = eye *G* = gills, *L* = lens, *Nas* = nasal placode *Ot* = otocyst, *Stom* = stomodeum.

The stain on the eyes will be visible from the outside Fix the embryo in 10 per cent formaldehyde Shortly after fixation carefully dissect the head in an operation dish with Permoplast ground Remove the epidermis with a glass needle and slit the brain open at the dorsal side. Determine the extent of the stained area in the brain and the eyes If the original mark extended too far posterior then the floor of the forebrain and *midbrain* may be found stained It is surprising to find that the area in the medullary plate from which both eyes originate is one uniform, median region not separated by a piece of prospective brain During neurulation the mark will gradually expand to the sides and become dumbbell shaped The lateral parts will be folded up and come to lie in the lateral walls of

the forebrain from where they will be evaginated as optic vesicles The narrow median part of the mark will persist in the optic stalks. The part of the brain which separates the eyes in later stages is derived from material which was located posterior to the eye area in the medullary plate and which has moved forward during neurulation These findings have been of great importance in the interpretation of the origin of Cyclopia, a malformation in which one single median eye, instead of two eyes, is found (see Adelmann, 1936)

Experiment 6 Vital Staining of the Prospective Nasal, Balancer, Lens, Gill, Ear Ectoderm

(Carpenter 1937)

Procedure

Remove the jelly membranes but not the vitelline membrane Mount the neurula in a depression in an operation dish, so that the prospective head region points upward Press Permoplast from the edge of the groove gently against the embryo to hold it tightly in position Stain one of the areas listed above using Figure 17 for your orientation Make several experiments Follow the shifting of the marks during neurulation and make sketches of transitional stages and of the position of the mark in stages H29–H35

Experiment 7 Vital Staining of the Border between Head and Trunk

(Manchot, 1929)

Procedure

Place a mark in the middle of the medullary plate at the point where the folds come closest together or place the mark at the same level on the left or right prospective epidermis outside of the medullary folds (*B* in Fig 17 *c*) Allow the embryo to develop to at least stage H24 Identify the position of the mark, dissect if necessary Note the number of the somites in front of the mark Visualize that about two-thirds of the medullary plate in the early neurula is prospective head and only one third is prospective trunk and tail The latter part stretches enormously in tail bud stages the tail originates largely by growth from the tail bud

e) Vital Staining of Lateral-Line Placodes

(After L S Stone)

Aquatic vertebrates, including amphibian larvae, possess a special type of sense organs—the lateral line organs which are receptors for water pressure and aid the animal in its orientation in flowing water They are cup-

shaped structures composed of sensory and supporting cells and are exposed to the surface. They are arranged in lines which form specific patterns on the head trunk and tail Those of the head are innervated by a special branch of the nervus facialis those of the trunk and tail by a branch of the nervus vagus

Their mode of origin is unique in several ways. The three trunk and tail lines characteristic for urodele larvae originate from ectodermal thickenings, or placodes which are part of the vagus system and are located immediately behind the otocyst The deeper cells of these placodes become detached and migrate caudad in a body They glide along the inner surface of the epidermis in three distinct columns forming one dorsal

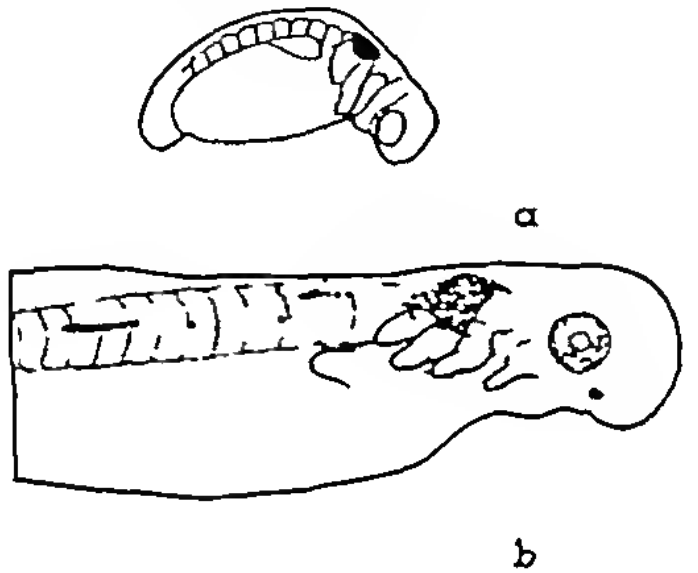

Fig 8 —Vital staining of the lateral line placodes (from Stone 1933) See text

one middle and one ventral line On their way they deposit at regular intervals clusters of cells which differentiate into the cup-shaped sense organs The latter push through the ectoderm and are thus exposed to the surface. All placode material is used up when the migrating primordia have reached the caudal end of the tail. The primordium of the sense organs is accompanied by the lateral line branch of the vagus nerve which originated at about the same time and in a fashion similar to that of the sense organs i e from a vagus placode which, however was supplemented by neural-crest material Side branches of this nerve innervate each individual sense organ The lateral line organs of the head and their nerves originate in a similar fashion from placodes belonging to the facialis system

The mode of origin of the lateral line sense organs is of particular interest because it demonstrates long range directional migration of cell

groups along specific paths This process can be observed on the living embryo with the aid of vital staining The intriguing problem of the determination of the lateral line pathways has instigated the classical experiment of Harrison (1904) in which parts of darkly pigmented embryos of *R sylvatica* were combined with parts of the lighter *R. palustris* embryo and the deposition of dark sense organs on the light epidermis was observed This was one of the first instances in which the method of heteroplastic transplantation (previously worked out by G Born) was applied in an analytical experiment. Stone has continued this analysis using the methods of transplantation of placode primordia and of vital staining His papers (1922, and particularly 1933) should be consulted

Experiment 8

Material

Amblystoma—any species stages H28–H30
operation dish
agar stained with neutral red
glass bridges
lily cups

Procedure

1 Remove all membranes and place the embryos in an operation groove, right side up

2 Locate the otocyst above the second visceral arch Place a piece of red agar on the epidermis covering the otocyst and the region immediately behind it (Fig 18, *a*) Hold the agar in position with a glass bridge Press tightly

3 Stain for 15–25 minutes until the epidermis is stained deeply red

4. Remove the glass bridge and transfer the embryo to a Lily cup

5 Make a sketch of the head indicating the stained area

6 Observe the embryo twice a day for several days under high power of the binocular Watch the middle line grow out horizontally across the middle of the somites It is an elongated, club-shaped structure which moves backward and leaves behind on its path darkly stained spots, the lateral line sense organs. A similar though smaller red mass—the primordium of the dorsal line—grows out somewhat later It turns dorsad and follows the upper border of the somites (see Fig 18 *b* and Stone, 1922 Figs 1–12 also Stone, 1933 Figs. 1–3) Make careful sketches

Further Suggestions

7 Students who wish to observe the finer details of the differentiation *in vivo* should construct an observation chamber (Stone 1933 pp 510 f),

which allows observations on the narcotized animal under the compound microscope

8 The development of the lateral lines of the head may be studied in a similar way by staining the preauditory facialis placode

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3 SOME ANALYTICAL EXPERIMENTS IN PREGASTRULATION STAGES

a) ARTIFICIAL PARTHENOGENESIS IN THE FROG BY PRICKING

(After Parmenter)

Fertilization that is the union of the two gametes has two important consequences By the fusion of egg and sperm nuclei the diploid chromo-

some number is restored, and two different sets of nuclear hereditary factors are combined Furthermore, fertilization activates the first cleavage and thus initiates development

The occurrence of *parthenogenesis* (i.e , development of the egg without insemination) in a number of animals—for instance, in rotifers, aphids, Cladocera and the honeybee—shows that neither the fusion of two cells nor the fusion of two nuclei is a prerequisite for the initiation of development In 1901 Jacques Loeb made the discovery that the sea urchin egg can be stimulated to develop into a normal larva without fertilization, by placing it in hypertonic sea water ('artificial parthenogenesis) The conclusions drawn from normally parthenogenetic eggs can thus be extended to eggs which normally require the sperm for activation Many different chemical and physical agents are now known to activate eggs—for instance surface active substances like fatty acids which have a slightly cytolyzing effect, hypertonic and hypotonic salt solutions, temperature changes, irradiation and even pricking with a fine needle In addition to the sea urchin and starfish eggs those of several annelids, of mollusks of the frog and others, have responded to such treatments Students who are interested in the theories which have been advanced to account for the activating role of the sperm on the basis of artificial parthenogenesis experiments are referred to J Loeb (1913), Wilson (1925), Spek (1931), Just (1939), Tyler (1941)

Artificial parthenogenesis in the frog's egg —The frog's egg is rather refractory to chemical agents, but in 1910 Bataillon discovered that artificial parthenogenesis may be obtained by puncturing the egg with a fine needle of glass or platinum However this treatment is successful only if the needle is dipped into frog's blood and a small amount of blood is introduced into the egg The role which the blood plays has not been explained satisfactorily but its indispensability has been confirmed by all subsequent observers A small percentage of parthenogenetic frogs develop into tadpoles and Loeb, Parmenter and others have succeeded in raising a number of these through metamorphosis to sexual maturity (see photographs in Loeb 1921)

The chromosome situation in these specimens is of particular interest. Parmenter (1933 1940) found a number of young tadpoles to be haploid but all those which had metamorphosed were diploid According to Parmenter the regulation of the chromosome number may occur even before cleavage starts It will be remembered that in the frog the egg is in the stage of the second maturation spindle when insemination takes place In cases of experimental parthenogenesis the spindle may be withdrawn into the egg and the formation of the second polar body suppressed so

that the egg starts with a diploid number of chromosomes (all derived from the female pronucleus) Or both polar bodies may be formed but the first nuclear division may not be followed by a cytoplasmic division

Experiment 9

Material

2 ovulating females of *R. pipiens* or other frog species
1 nonovulating female as a source of blood
10–12 clean slides
2–3 pipettes
Petri dishes or finger bowls
1 Syracuse dish
3–4 very fine glass needles
paper towels
spring or pond water

Sterilization —In this experiment it is imperative that contamination with sperm be avoided Sterilize glassware, etc. in the autoclave and wash your hands with 70 per cent alcohol wash the frogs under running water Have no male frogs in the laboratory It is also advisable to autoclave all water to be used in this experiment.

Procedure

1 Prepare the blood of the nonovulating female as follows Wash the female and pith it. Open the abdomen and expose the heart. Cut off the tip of the ventricle and allow the blood to accumulate in the pericardial cavity or in the coelom Close the abdominal skin flaps until you are ready to use the blood

2 Strip eggs on sterile slides (p 34) Strip 2 rows of eggs onto each slide. Prepare 6–10 slides in this way

3 Smear eggs with blood. Dip a piece of muscle into the blood pool of the frog previously prepared and smear all eggs with blood. Be careful not to exert any pressure on the eggs

4 *Pricking* —Under appropriate illumination prick each egg with the glass needle somewhere within the animal hemisphere The germinal vesicle is usually under the animal pole. It should not be injured. Prick gently but be sure that the tip of the needle has definitely entered the egg Blood corpuscles must be taken in with the needle. The cortical damage should not be extensive. On 1 or 2 slides leave the eggs unpunctured use them as controls and mark them as such.

5 As soon as the eggs have been pricked, immerse all slides, including the controls, in sperm-sterile water

6 After 30 minutes, when the jelly is swollen gently separate the eggs from the slides by means of a sterile scalpel. Keep experimental and control material strictly separate.

7 *Observations* —The first cleavage is to be expected about 2¾ hours after pricking (at 18°–20° C) Distinguish normal and aberrant cleavage Make sketches of both.

8 After 6–8 hours remove all noncleaving eggs Count and calculate the percentage of cleaved eggs

9 *Further observations* —Keep careful record of all eggs which continue to cleave Isolate those which begin to gastrulate and follow their development Compare parthenogenetic with normal tadpoles

Note —You may expect 5–10 per cent cleavage at best

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b) ALTERATION OF THE CLEAVAGE PLANE BY PRESSURE

(After G Born)

Some of the earliest experiments in embryology were inspired by Weismann s theory of development and heredity (see Weismann, 1892) This theory was the first to attribute a decisive role in organ determination to factors located in the chromosomes These factors which are equivalent to our genes were called determinants by Weismann The cytoplasm he considered merely as building material He construed an ingenious scheme by which the determinants would be distributed over the different areas of the developing embryo According to his hypothesis of qualitative nuclear division the two daughter cells of a dividing cell

would obtain qualitatively different assortments of determinants and in this way the germ plasm would be broken up into its units by successive mitotic divisions. For instance in cases where the first cleavage plane coincides with the median plane of the future organism one blastomere would obtain all determinants for 'left organs and the other all determinants for "right organs In a later step the determinants for neural structures would be segregated from those for epidermis and so forth, until each cell would be left with one determinant, which would then be instrumental in its structural differentiation Weismann s theory is thus the prototype of a preformistic theory—more specifically of a nuclear preformation It is now abandoned as a theory of embryonic differentiation, because we have ample evidence that in mitotic nuclear divisions both daughter cells receive quantitatively and qualitatively equal chromosome materials However, parts of Weismann s theory are incorporated in the present gene theory of heredity Moreover his theory of differentiation challenged the most outstanding embryologists of the turn of the century and inspired several classical experiments which are now cornerstones of experimental embryology—for instance, Driesch s experiment of separating the blastomeres of the sea-urchin egg Roux's famous pricking experiment on the frog's egg and Spemann s constriction experiment (see p 69)

The experiment of ' cleavage under pressure was devised by Driesch (1892) to test the validity of this theory In the sea urchin egg and in the frog s egg the first two cleavage planes are meridional, but the third is equatorial. If the eggs are mounted on a glass plate animal pole upward, and then slightly compressed by placing another glass plate on top of them, the third cleavage plane will also be meridional. If the pressure is released at the 8-cell stage the fourth cleavage plane will be horizontal This procedure does not change the arrangement of the cytoplasmic structure, but it results in a complete reshuffling of the nuclei (see Weiss 1939 Fig 33 p 200) Some nuclei which in normal development would be located in dorsal organs now find themselves in a ventral position and should according to Weismann determine dorsal structures at the wrong place Generally speaking a completely disorganized patchwork of structures should result, if the hypothesis of unequal nuclear division were correct Instead normal embryos developed which proves that the blastomere nuclei cannot be qualitatively different The orderly pattern of differentiation must be brought about by other mechanisms Driesch experimented on the sea-urchin egg The experiment was repeated successfully on the frog s egg by G Born (1893) and O Hertwig (1893)

Material

fertilized eggs of *R. pipiens* or other species (artificially inseminated [p 30])
scalpel
10 slides
Permoplast
finger bowls
Lily dishes

Procedure

1 Obtain fertilized eggs by artificial insemination (p 30)

2 Clean and dry 10 microscope slides thoroughly Place narrow strips of Permoplast near both ends of 5 slides and parallel to the short edges. The strips should not be much higher than the diameter of the eggs and the membranes.

3 About 1 hour after artificial insemination when the jelly membranes are swollen cut out 30–40 individual eggs, using knife (scalpel) and forceps. Be sure to pick out fertilized eggs. Eggs whose vegetal (light) poles face lateral or upward are usually not fertilized On each slide with Permoplast strips place 4–6 eggs separately, each in a small drop of water Allow time for the eggs to assume their normal position, animal pole upward Set 10–15 eggs aside, as controls

4 Place a second slide over the eggs so that the slides are held together by Permoplast. Press the ends slowly but firmly with your thumbs until the eggs are flattened Control this procedure under the binocular microscope

5 Watch the appearance of the first cleavage, 2–2½ hours after fertilization The second cleavage will follow ½ hour later (The times for other amphibians are different.)

6 Observe the appearance and the plane of the third cleavage which is perpendicular instead of parallel to the plane of the slides Make sketches of several such 8-cell stages and compare with normal 8-cell stages from the same batch of eggs

7 When all or most of the eggs on a slide have reached the 8-cell stage release the pressure cautiously lift the upper slide by pushing two pointed instruments through the Permoplast strips on each end Discard the abnormal eggs and place all others In a finger bowl or a Lily cup Ascertain that no horizontal cleavage plane is present by turning the eggs sidewise

8 Label the dishes and protocol as follows

PRESSURE EXPERIMENT

March 22d 1 00 P.M. fertilized
2 00 P.M. 30 eggs pressed

3 00 P.M. first cleavage in 28 eggs
3:40 P.M. third cleavage plane meridional in 23 abnormal, 5
etc.

9 One half to 1 hour later observe the appearance of the first horizontal cleavage planes.

10 On the following days make observations on the further development of compressed eggs Those which develop normally are of course of special importance for the problem under discussion Make protocols sketches formulate in your own words the implication and the results of the experiment

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c) THE PRODUCTION OF TWIN EMBRYOS AND OF DUPLICATIONS IN *Urodela* BY CONSTRICTION

(After Spemann)

Few experiments have influenced the course of experimental embryology more deeply than Driesch's experiment on the sea urchin egg in which embryos were produced out of 1 egg by isolating the first 2 blastomeres This experiment demonstrated an unexpected regulative property in early developmental stages of an organism which shows little regenerative power in adult life This result was at once interpreted as a strong argument against any mosaic theory of development and paved the way for our modern epigenetic concept of development Because of its fundamental importance it was repeated on the eggs of many other invertebrates and vertebrates in many instances with the same result

In the urodele twin embryos and duplications can be obtained best by constricting the 2-cell stage in the plane of the first cleavage using a child's hair (Spemann 1901 1902 1903 and others) Spemann's papers give an exhaustive analysis of the results If the 2 blastomeres

were separated completely by a deep constriction, then the following results were obtained In a certain percentage of cases 2 whole embryos, that is "identical twins, developed They were small in size but otherwise normal In a larger percentage, only 1 blastomere gave a normal embryo whereas the other half remained an unorganized, though viable, spherical structure. The explanation is as follows In the first case the first cleavage plane (plane of constriction) coincided with the future median plane of the embryo In the second instance the first cleavage plane separated future dorsal from future ventral structures, and only the dorsal half gave a normal embryo The unorganized sphere of cells was therefore called "belly piece (*Bauchstück*) This implies that, in the salamander the first cleavage plane and the median plane of the embryo have no constant relation That this explanation is correct can be demonstrated by examining constricted eggs during gastrulation Some eggs will be found in which both isolated blastomeres develop blastopores and dorsal lips and these invariably give identical twins. In the majority of cases only 1 embryo has a dorsal lip and undergoes complete gastrulation and it is this half which develops into the complete embryo Spemann concluded that as early as the 2-cell stage the dorsal half differs qualitatively from the ventral half in that it contains "something" which enables it to undergo typical, proportionate differentiation. This "dorsal quality, which is lacking in the ventral half was identified later as the "organizer" and the constriction experiment may thus be considered as the first step in its discovery

When the two blastomeres are not separated entirely but constricted slightly so that they form a dumbbell shaped structure, anterior duplication duplicitas anterior may result (Spemann, 1903), resembling the two-headed monsters found occasionally in many vertebrates The degree of duplication depends on the degree of constriction slight constriction results in slight duplication of the head and such cases may even have a median eye in common deep constriction almost to the point of separation results in embryos which are fused together only at their tail ends. Strangely enough duplicitas posterior (monsters with one head and two posterior ends) never occurred in this experiment. Spemann explains this fact on the basis of the mechanics of gastrulation in constricted eggs (see Spemann 1938 p 159) Thus the constriction experiment contributes materially to an understanding of the origin of identical twins and anterior duplications

In some but not all cases of complete and incomplete twinning the symmetry relations of the internal organs (curvature of the heart and of the intestine position of liver gall bladder etc.) of one twin were found to be

inverted. This condition is known as "situs inversus viscerum et cordis" and is again identical with the same abnormality found occasionally in vertebrates In man this abnormality is very rare even in identical twins but it is the rule in laterally conjoined human twins (Newman 1940) It is significant that in the constriction experiments all left embryos showed normal situs and right embryos showed the mirror imaging The experiment of constriction thus broaches another important problem—that of the origin of bilateral asymmetry in vertebrates

EXPERIMENT II

Material

Triturus viridescens[1] or	child's hair
Tr pyrrhogaster	hair loop
2 watchmaker forceps	glass dish without Permoplast

Procedure

1 *Prepare the hair for constriction* —Select a fine hair hold it between the thumb and forefinger of your left hand so that it forms a loop as indicated in Figure 19 *a* Use the natural bend if it is curly Under the binocular microscope or loupe tie the upper end through the loop twice using the watchmaker forceps Then pull on both ends until the diameter of the loop is slightly larger than the smaller diameter of the oval capsule (2–2½ mm in the case of *Tr viridescens*) Cut both ends at some distance from the loop and submerge the loop in the operation dish (without Permoplast or paraffin bottom)

2 *Prepare the embryo* —*Triturus viridescens* females caught in the field during March and April will lay their eggs in captivity The eggs are folded between the leaves of *Elodea* The first cleavage occurs 8–10 hours after fertilization Eggs should be collected twice a day and watched at short intervals unless the laying has been observed The outer sticky membrane is milky In some eggs it can be peeled off easily and the egg is then visible inside the transparent capsule If one finds it difficult to remove the outer membrane, one may leave it intact.

3 *Constriction* —Wait until the 2-cell stage is well under way Hold the hair loop upright directly in the center of the visual field so that it appears as one streak. Hold it in place with one forceps and push the egg into the loop with the other forceps (Fig 19 *b*) When the loop is approximately around the middle of the egg capsule pull gently at its

[1] *Triturus viridescens* is preferable because in *Tr pyrrhogaster* the vitelline membrane frequently bursts under the inner pressure of the constricted capsule, resulting usually in the loss of one half (see Streett, 1940) In *Tr viridescens* both twins survive in a high percentage of cases.

free ends so that it fits loosely but does not yet constrict. Shift the loop until it is exactly in the middle of the capsule The slightest asymmetry will result in a conspicuous size difference of the egg halves after constriction, which will obscure the results Constrict the capsule slightly so that the egg is still free to move in the capsular fluid Again control the symmetry of the half-capsules, start anew if the halves are unequal Tilt the egg from one side to the other until the first cleavage plane is exactly under the loop Then pull slowly and evenly on both ends of the hair until the desired degree of constriction is reached (Fig 19, *c*)

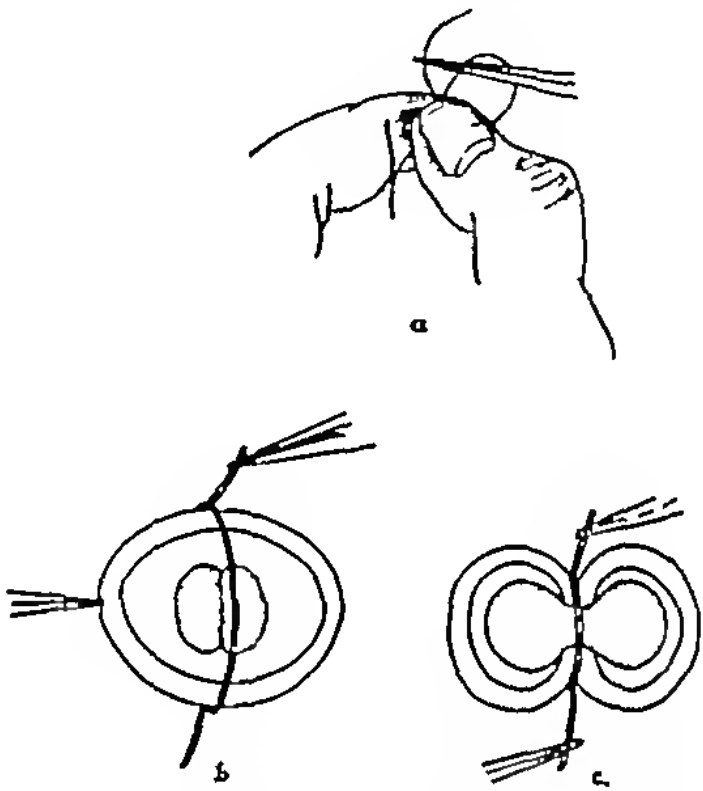

Fig 19 —Constriction of a urodele egg (2-cell stage) with a hair *a*=preparation of the hair loop *b*=placing of the egg in the loop *c*=constriction.

In order to obtain *complete twins* it is usually not necessary to constrict until the two halves are entirely separated Such a deep constriction usually results in the bursting of the vitelline membrane and the loss of one or both halves. If the bridge between the halves is narrow, it will break apart by itself after a few hours Another way of avoiding rupture of the vitelline membrane is to complete the constriction in several steps 10–30 minutes apart.

In order to obtain conjoined anterior duplications constrict only slightly

Note —The same results may be obtained by constricting in one of the planes of the 4-cell stage

4 After constriction is finished cut off the free ends of the loop near the egg Make a sketch indicating the degree of constriction (diameter of "handle in proportion to diameter of the lateral halves)

5 Place the egg in a clean dish.

6 Observe the gastrulation. It is indicative of the future result Twins will be obtained if the blastopore and upper lip are shared by both sides If you find gastrulation in one half only then you may conclude that the plane of constriction was frontal. The half which does not gastrulate will form a belly piece.

7 Observe further development and make sketches In the late tail bud stages the twins will be crowded in the narrow capsule and thus en dangered Try to grasp the hair loop with your sharpest forceps clip it, and remove it. In later stages, when the capsule has lost its turgor it may be removed easily However it is dangerous to do this in early tail bud stages because the embryos are liable to be squeezed to pieces when the highly turgid capsule is punctured If whole mounts of the constricted egg with the hair loop intact are desired fix the egg within the capsules in formaldehyde

8 Try to raise twins or duplications to stage H40 or older Observe the situs viscerum of the twins (see p 70)

FURTHER SUGGESTIONS MEROGONY

In 1914 Spemann discovered that salamander eggs can be constricted shortly after fertilization before cleavage starts. Urodeles are known to exhibit 'physiological polyspermy that is several spermatozoa enter the egg In normal development all but one disintegrate In constricted uncleaved eggs one half contains the egg nucleus and will subsequently develop with the diploid zygote nucleus The other half contains accessory spermatozoa and in the absence of the zygote nucleus, one of them will become activated In a few favorable cases a haploid embryo will de velop out of this combination of maternal cytoplasm and paternal nu cleus Embryos developing from egg fragments are called 'merogons those which develop with the sperm nucleus only are called 'andro-mero-gons Fankhauser and his associates have used this method for an analysis of cytological problems connected with polyspermy fertilization and haploidy (see Fankhauser 1937*a* and *b* and Fankhauser and Moore 1941)

Baltzer and his associates (in Bern Switzerland) have seized upon this unique opportunity of building up individuals of maternal cytoplasm and paternal nucleus and have inseminated such nonnucleated egg fragments with sperms of different species These experiments have given very im

portant clues as to the role of nucleus and cytoplasm in heredity and development (cf reviews in Baltzer 1933 1940, Hadorn, 1937) Students who were successful in the preceding experiment should try to obtain andro-merogons by constriction

Experiment 11*a*

Material

According to Fankhauser (1932), andro merogons of *Tr viridescens* do not develop beyond gastrulation, whereas those of *Tr pyrrhogaster* may develop into older embryos (Fankhauser, 1937*b* Streett, 1940) However the number of successful cases will be small

Procedure

If possible use eggs whose deposition was observed Notice an unpigmented area at the animal pole with a small black spot in its center The latter is the second polar spindle Constrict, as before, through animal and vegetal pole For interpretation of your results consult Fankhauser (1937*b*) and Streett (1940)

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d) SCHULTZE S EXPERIMENT PRODUCTION OF DUPLICATIONS BY INVERSION

(After Schultze and Penners and Schleip)

In 1895 O Schultze studied the role of gravity in frog development hy compressing eggs in the 2-cell stage between glass plates and then turning them upside down They were left in this position for several hours and then released from pressure If this is done part of the heavy yolk of the vegetal pole sinks down lighter parts of the egg cell move upward, and a complete rearrangement of the inner egg substances takes place whereas the cortical layer seems to remain rather stahle O Schultze made the unexpected discovery that a high percentage of *double monsters* resulted

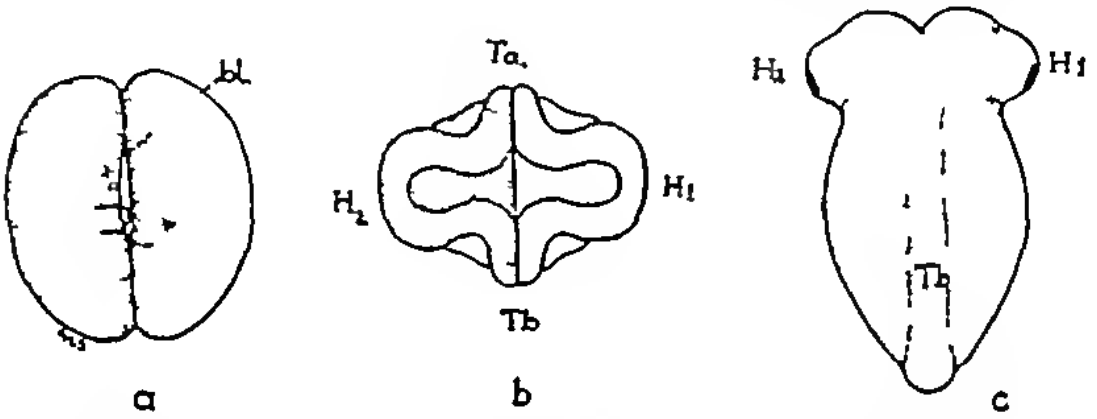

FIG. 20 —Duplicitas cruciata in the frog, produced by inversion of the egg *a* = gastrula stage *b* = medullary fold stage *c* = tail bud stage *bl* = blastopore *H H* = the two heads *Ta*, *Tb* = the two tails (modified after Schleip, 1929)

from this procedure Penners and Schleip (1928*a* and *b*) and Pasteels (1938) repeated the experiment on a large scale and ohtained the same re sults These duplications undoubtedly owe their origin to the shift of yolk and other materials which in turn causes great disturbances of the gastrulation process As a result in many instances two upper lips in stead of one are formed which give rise to two separate axial-organ sys-tems. One type of duplication is of particular interest the so-called duplicitas cruciata It derives its name from the crosslike appearance of the duplicated structures In this strange monstrosity two heads are opposite each other and so are the two trunks and tails but the plane of symmetry of the heads is perpendicular to that of the trunks and tails (Fig 20 *b c*) This duplication originates apparently from an elongated blastoporal groove (*bl* in Fig 20 *a*) the edges of which behave as upper lips Only a few embryos will be found to represent duplications of the diagrammatic clearness of Figure 20 Most of them are asymmetrical and distorted However all the possible modifications of this and other types

have been catalogued and analyzed by Penners and Schleip (1928) The student should consult the illustrations of their papers to identify his material. It is impossible to discuss here the far reaching theoretical implications of this experiment The student is referred to the papers *quoted* above and to Schleip (1929) and Dalcq (1938) The experiment is presented here because it demonstrates by means of a very simple technical procedure a fundamental fact of experimental embryology that more than one embryo can develop from one egg

Experiment 12

Material

fertilized, uncleaved eggs of *R. pipiens* (see p 30)
microscope slides
Petri dishes or finger bowls
Permoplast

Procedure

1–3 As in Experiment 10 (alteration of cleavage plane, p 68)

4 Place the second slide over the eggs and press gently so that the jelly membranes are compressed but the eggs remain movable

5 When the first cleavage is well under way i.e , 2–3 hours after fertilization (in *R. pipiens*) press the ends of the upper slide firmly with your thumbs until the eggs are flattened Control this under the binocular microscope turning the slides edge up

6 Turn all slides upside down while the eggs are in the 2-cell stage. Do not place them in water The eggs should be pressed so tightly that the white poles remain uppermost. Eggs which rotate back or those whose axes are not exactly perpendicular to the slides must be discarded later

7 After 10 minutes submerge all double slides in water in the inverted position use Petri dishes or finger bowls

8 During the following hours watch the flow of light and dark materials Make sketches

9 After 5–24 hours remove the eggs from the slides and place those embryos which are in good condition in a finger bowl or a Lily dish.

10 Study the gastrulation and subsequent development, isolate the best cases and protocol them individually Make sketches The late neurula stage with raised neural folds is the most interesting stage, because it shows the general structure (symmetry relations etc.) of duplications more clearly than later stages do

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e) CENTRIFUGING

(After Jenkinson)

Centrifuging of eggs has been one of the standard methods of experi mental embryology since the beginning of this century Centrifuging with sufficiently high force results in a redistribution of the formed inclusions of the egg cytoplasm For instance in the centrifuged frog's egg three distinct layers can be seen a yellow cap of oil and fat globules at the centripetal pole a colorless middle layer composed of proteins water salts etc. and a third layer containing yolk and pigment granules at the centrif ugal pole It is of considerable interest to find out whether or not the re arrangement of these egg substances affects the differentiation of the em bryo Many invertebrate and vertebrate eggs have been centrifuged at different stages and with different forces (comprehensive review in Mor gan [1927] chap xxii) The essence of all these experiments is that high speed centrifuging will result in abnormal development but for most eggs a moderate rate of rotation can be found which will result in an abnormal stratification of the egg inclusions yet will not interfere with nor mal development For instance, fat globules or pigment granules may be accumulated in abnormal concentration in the nervous system of a tad pole and be missing in other organs where they are normally found. An important conclusion can be drawn from these experiments these formed cytoplasmic inclusions cannot be considered as organ-determining or formative substances in the sense that their presence in a given organ primordium is necessary for its normal differentiation However one should not forget that the cortical layer of the egg is in all probability rather stable and not disturbed by centrifuging and that there may exist in the cytoplasm a micellar framework which also may remain more or less unaffected by heavy centrifugal forces Therefore it would be unwar ranted to conclude that there exists no preformed structuration whatsoever in the egg All we can say is that its visible components and their distribution have nothing to do with the future organization of the em bryo

In batches of centrifuged eggs there always will be found numerous monstrosities of all kinds, for instance double monsters multiple tails headless monsters spina bifida, etc. (Jenkinson, 1915, Banta and Gortner 1915 Beams King and Risley, 1934) This is not surprising since the dislocation of the yolk and of other substances will, in many instances, interfere with normal gastrulation etc and also a dislocation of the organizer material may be expected

Centrifuging is an effective way of segregating the different constituents of the egg The different layers actually can be separated and subjected to a chemical and physicochemical analysis The application of the ultra-centrifuge which provides a much more efficient tool for investigations along this line, has already brought a revival of interest in centrifuging experiments

Experiment 13

Material

uncleaved eggs of *Rana* (any species) obtained by artificial insemination (p 30)
any type of electric centrifuge
finger bowls
large mouthed pipettes

Procedure

1 Use eggs ½–1 hour after fertilization when the jelly membranes are swollen Do not remove the latter Fill the tubes of the centrifuge with eggs do not crowd them

2 Run several series of experiments Vary the speed (between 1,500 and 3 000 revolutions per minute) and the time of exposure (5–10 minutes) Take careful protocols and keep the different batches in separate carefully labeled finger bowls. Find the optimal procedure for the particular centrifuge and the species of frogs which you use

3 Observe under the microscope the redistribution of materials (consult Jenkinson 1915)

4 The first cleavage is to be expected 2½ hours after fertilization (in *R pipiens*) Calculate the percentage of normal cleavage and discard the noncleaving eggs

5 Observe gastrulation and young tadpoles 2–5 days later Take protocols Try to identify the malformations

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4. TRANSPLANTATION EXPERIMENTS TO DEMONSTRATE SELF DIFFERENTIATION"

a) INTRODUCTORY REMARKS. TERMINOLOGY

Before starting transplantation experiments the student should read the chapters on 'determination' in one of the textbooks of experimental embryology.

Experiments in which the first 2 blastomeres were isolated or inverted (pp. 69–75) have shown that each blastomere can produce a whole embryo. In the terminology of Driesch, its 'prospective potency' is greater than its 'prospective significance' (actual fate in normal development). This implies that the destiny of a given part of the embryo in the 2-cell stage is not yet definitely fixed or "determined". It should therefore be possible to interchange embryonic materials during early stages without disturbing the normal development. On this reasoning are based the classical transplantation experiments of Spemann (1918) in which prospective epidermis and prospective medullary plate of the early gastrula of a urodele were interchanged. The result was that prospective epidermis formed part of the nervous system and prospective medullary material formed part of the epidermis. Thus it was shown that in early gastrula stages these embryonic areas are not yet irreversibly fixed or "determined". They differentiate in accordance with their new environment (*ortsgemäss*). In other words, their fate is being determined during or after gastrulation by factors which reside in the tissues outside of the transplant ('extrinsic' factors). It was found in later experiments that the subjacent mesoderm 'determines' the medullary plate during gastrulation.

If a similar experiment of *heterotopic transplantation* (i.e. transplantation to an abnormal position) is done in stages after gastrulation many parts will differentiate according to their prospective significance in disregard of their new environment, as if they had been left in their normal place. For instance, the prospective eye, brain or limb areas of the neurula stage will form eye, brain or limb when transplanted to the flank. W. Roux coined the term self-differentiation for this capacity. Obviously a change has taken place in these primordia during gastrulation. They have acquired independence of their environment and now contain within themselves all factors which are essential for their further differ-

entiation From now on they are "determined and one may properly call them eye brain, or limb *primordia* although they are not yet visibly different from one another

The terms "self-differentiation' and "determination must be qualified precisely whenever they are used otherwise they are ambiguous and misleading In applying the term "self-differentiation to an embryonic area it is necessary to state at what stage the latter has acquired its self differentiating capacity For instance the eye forming area is self differentiating *from the neurula stage on* Furthermore, self-differentiation is always relative Absolute self-differentiation does not exist an organ primordium remains dependent on its environment in certain respects until its development is completed It is necessary to state what specific structural features are to be tested with respect to their self differentiation or dependent differentiation because a primordium may be self-differentiating in one aspect of its development but, at the same time be dependent on extrinsic factors in other aspects of its differentiation For instance in the early tail bud stage of a salamander a certain area in the flank will differentiate into a forelimb when transplanted heterotopically it is self-differentiating from that stage on as far as its general morphogenesis is concerned However experiments of Harrison have shown that the same primordium at the same stage is not yet irreversibly determined with respect to its 'laterality this primordium may develop into a right or a left forelimb depending on the site of implantation It is self-differentiating as a whole but still depends on extrinsic factors for its symmetry relations In Experiment 14 the following question will be raised Is the balancer primordium which is self differentiating as a whole from tail bud stages on dependent on, or independent of its adjacent structures with respect to its direction of outgrowth and the time of its resorption? Likewise the central nervous system is blocked out roughly in the medullary plate stage It will differentiate into nervous tissue and even into special parts (forebrain spinal cord etc) when transplanted heterotopically However many structural details are not yet 'determined at that stage but become fixed in later stages under the influence of factors extrinsic to the nervous system For instance the size of the spinal ganglia and the number of neurons in the motor column are controlled by the developing peripheral structures to be innervated the extirpation of a limb primordium results in a size reduction of the limb-innervating ganglia and motor column In this respect the nervous system is not self-differentiating but remains under extrinsic control up to a remarkably late stage of differentiation In other words determination is not a single act but a process of gradual emancipation

from extrinsic factors Many other instances of the relativity of self differentiation will be illustrated in the following experiments

In the analysis of the process of determination the following three questions are pertinent

1 At what *stage of development* does a given embryonic area become relatively self differentiating that is independent of certain extrinsic factors?

2 Which *developmental processes or structural details* (e g., morphogenesis of the whole organ, its symmetry relation its quantitative growth) are self-differentiating at a given stage?

3 With respect to what *extrinsic structures or factors* (e g adjacent flank tissue, innervation, hormones) is the primordium under discussion independent or dependent?

The following experiments illustrate relative self-differentiation of whole organ primordia. They are designed to stress these points (1) to make the student acquainted with the method of transplantation (2) to illustrate the fact that in the neurula and tail bud stages of amphibians many organ forming areas are self-differentiating units as far as their gross morphological differentiation is concerned and (3) to impress the student with the fact that the process of determination involves changes in the in visible properties of embryonic materials The primordia to be transplanted are in no way distinguishable from adjacent areas yet they be have differently in transplantation experiments

The balancer primordium was found to be the most favorable object for the first exercise in transplantation It is easy to locate and easy to han dle The transplant shows visible differentiation two days after operation and no dissection or sectioning is necessary for its identification Further more the balancer offers an excellent opportunity for the study of inter esting side issues For instance the question of whether the direction of its outgrowth is determined by intrinsic factors or by the surrounding host tissue may be analyzed by varying the orientation of the transplant. Likewise the question of whether the time of its resorption is determined by intrinsic factors or by the host may be studied by using hosts and donors of different ages

b) TRANSPLANTATION OF BALANCERS IN *Urodela*
(After Harrison)

The balancers are a pair of slender rod like appendages which project from the side of the head a little behind and below the eyes and which serve as props to hold the head off the bottom and to prevent the larva from falling over on its side until the forelegs develop and assume that office (Harrison 19 4 p 349)

They are characterized by a club-shaped thickening at their ends which

secretes a sticky mucus This "secretion cone' and its stickiness should be used as a criterion for the identification of transplants as "balancers" Transplants which do not show it may be rudimentary balancers or merely epidermal outgrowths Stickiness can be tested easily with a hair loop The balancer is innervated by a fine nerve and vascularized by a small artery and a small vein Its rigidity is maintained by the firm "balancer membrane' which is located at the base of the epithelium

In *A maculatum* the balancers become visible externally in stage H34 they begin to secrete mucus in stage H38 and reach their full size in stages H40 or H41 The balancers are transitory larval organs which have only a short life span In *A maculatum*, regressive changes (constriction at the base) begin at stage H45 Soon afterward the entire balancer is shed by breaking off at the base In *A opacum* the balancer disappears in a different fashion It shrivels, beginning at the tip, and is largely resorbed The remnant seems to be cast off as in other forms Balancers are found only in certain species of urodeles, for instance, in most species of *Triturus* in *A maculatum jeffersonianum*, *opacum*, and *microstomum* but not in *tigrinum* (except for a few local strains in which rudimentary balancers were described by Nicholas 1924)

Students should study the structure development and disappearance of normal balancers before starting experimental work Harrison (1924) should be consulted for all details concerning their development and histological structure Kollros (1940) for details concerning their disappearance

The experiment is supposed to demonstrate the self-differentiation of the balancer primordium after it is determined but before it is visible Harrison (1924) has found that when prospective balancer ectoderm in stages H28 or younger is transplanted to the head of another embryo it will form a balancer but that when transplanted to the flank it will not do so The same ectoderm taken from stages older than H28 will grow out to form a balancer in any region Harrison concluded that up to stage H28 prospective balancer epidermis is not entirely self-differentiating but still dependent on the underlying head mesoderm Therefore in order to obtain balancer formation it is necessary to transplant young epidermis to the head or young epidermis *plus* underlying mesoderm to the flank old epidermis will give balancer in any position

Experiment 14

Material for Experiments 14 14a–b

Ambystoma maculatum or *opacum* (*A tigrinum* has no balancers) in stages H28 H32

standard equipment (p 41)

Procedure

1 Select two embryos of approximately the same stage remove all membranes including the vitelline membrane (see p 37) Fill the operation dish with full-strength Holtfreter solution

2 Transfer both embryos in a wide mouthed pipette to the operation dish (Syracuse dish with smooth Permoplast ground) Dip the pipette under the surface of the water before releasing the embryo

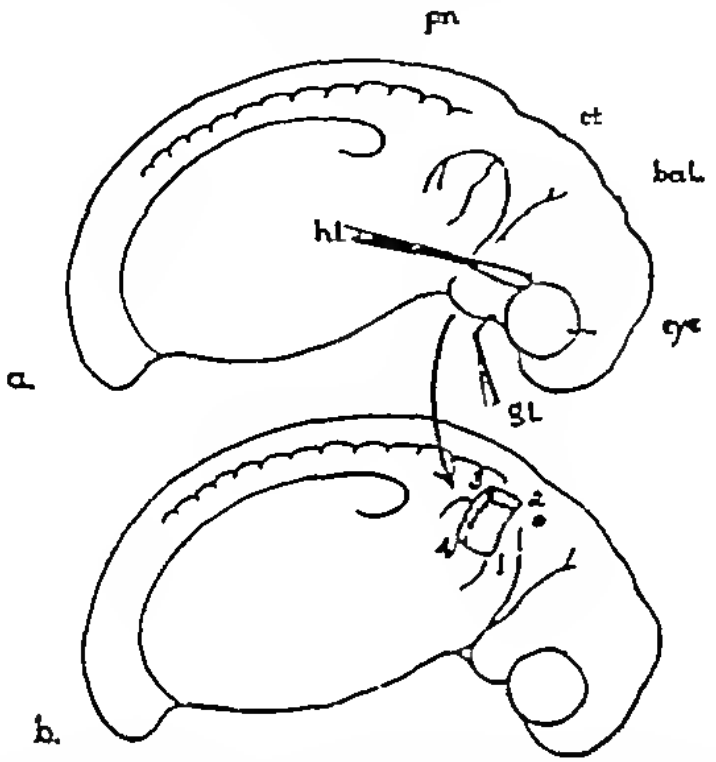

FIG 21 —Balancer transplantation *a* = donor embryo glass needle (*gl*) and hair loop (*hl*) in the position in which they are held during the extirpation *bal* = balancer region *ot* = otocyst *pn* = pronephros *b* = host embryo. 1 2 3 4 = hole prepared for the reception of the transplant (see text)

3 With a glass rod with ball tip make two grooves one beside the other of such size and shape that the embryos will fit into them when lying right side up Carefully smooth the edges Place the embryos in the grooves right side up

General rule Operate on the right side only use the left side as a control

4 *Prepare the host embryo* —Choose the site of implantation either the trunk region just below and close to somites 6–8 (behind pronephros not too far ventrally) or the head region in the level of and immediately behind the otocyst (Fig 21 *b*) By means of the glass needle cut out a rectangular area of ectoderm approximately the size of the optic vesicle To do this pierce the ectoderm with the point of the needle push the

needle gently forward underneath and parallel to the ectoderm and pierce again at the upper corner of the desired hole as if you were making a stitch in sewing (line 1–2 in Fig 20, *b*) Stroke the hair loop gently against the needle until the ectoderm is cut. Do the same on the other three edges first line 4–3 then line 1–4 and line 2–3 Lift out and discard the ectoderm and some of the underlying mesoderm

5 In order to assure the right orientation of the transplant in the host embryo, it is advisable to stamp a vital stain mark on the upper right corner of the transplant Before starting the extirpation, place a small piece of red or blue agar on the upper posterior end of the right eye and adjacent regions of the donor embryo Press it against the embryo with a glass bridge leave it there for 10–15 minutes

6 *Extirpate the prospective balancer region from the donor embryo* (*bal in Fig 21, a*) —This region lies posterior and slightly ventral to the eye on the mandibular arch The dorsal border is on a level with the dorsal margin of the eye, the ventral border is slightly above the mid ventral line and parallel to it. The anterior limit is along the posterior margin of the eye The posterior limit is behind the first gill slit Cut these four edges with the glass needle and hair loop (as under sec. 4) Remove ectoderm and mesectoderm very gently transfer the piece on the tip of the needle or with the hair loop to the host embryo and place it temporarily on the Permoplast ground near the host embryo Notice the mesodermal cells attached to the inner side of ectoderm In transplanting try to retain the proper orientation of the transplant with respect to the longitudinal axis of the host embryo do not rotate it

7 *Implantation* —It may be necessary to enlarge the hole in the host embryo by pulling the edges apart or by removing some mesenchyme cells with the tip of the needle With hair loop or needle lift the transplant into the hole Make the edges fit and place a glass bridge of proper size over the transplant so that it covers the transplant completely Work as quickly as possible since the transplant is likely to curl Try to implant in undisturbed orientation If the transplant was rotated intentionally or unintentionally note this in your protocol immediately after operation Press the glass bridge into the Permoplast ground until it holds the transplant firmly in place Too strong pressure may cause disintegration of the transplant

8 After 20 minutes lift the glass bridge gently remove the loose cells adhering to the edges of the transplant using the tip of the glass needle or the hair loop If the transplant has not healed in completely place the glass bridge back for 15 20 minutes. Otherwise lift the embryo out of the groove using the hair loop and clean it of all adhering cells and Permo-

plast. Transfer it very gently in a wide monthed pipette to a section dish or a Lily cup half filled with $^1/_6$ Holtfreter solution Dip the pipette under the surface before releasing the embryo Place the embryo right side up

9 Clean the donor embryo and transfer it to the same dish right side up

10 Give the embryos a serial number (e g *bal* 1), label the dish, and prepare a *protocol* Carefulness in drafting the protocol is as important as carefulness in the operation. Make sketches of donor and host indicating the position orientation etc , of the transplant.

Example of protocol

bal. 1 Ambystoma punctatum

March 11 *donor* stage H30
host. stage H31
trpl right balancer ectoderm (refer to sketch) a few mesoderm cells ad here to ectoderm
implanted ventral to somites 7–9 (refer to sketch) in normal orientation healed after 30 minutes
donor saved wound beginning to heal

March 13 *donor* stage H35 OK left balancer just visible wound on right side healed
host. stage H36 OK both host balancers just visible
trpl. healed first beginning of outgrowth, in typical direction etc.

Note all changes in the transplant particularly time and direction of the outgrowth of the transplanted balancer Compare with the control balancer of the donor or of normal embryos of the same stage as the donor

Make 3–4 operations choose different sites of implantation

Note —In rare instances the transplant will form a double balancer Occasionally the tissue which healed over the wound of the donor will regenerate a balancer

EXPERIMENT 14a

Rotate the transplant 90 or 180° —Observe carefully (and sketch) the direction of outgrowth of the balancer compare it with the normal balancer In order to assure the desired orientation of the transplant it is essential to vital stain its upper right corner before excision as under section 5 Is the direction of outgrowth determined exclusively by intrinsic factors or is it influenced by the host?

EXPERIMENT 14b

Obtain embryos of different stages —Transplant from a young donor onto a host which is several stages older and vice versa Operate with great

care in order to keep the donor alive Observe the first appearance of the transplanted balancer and compare it with that of the left donor (control) balancer Likewise observe the time of shedding in the transplant, the host and the donor (control) balancer Is the life-cycle of the transplant in any way influenced by the host? (See Kollros, 1940)

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c) TRANSPLANTATION OF FORELIMB PRIMORDIA

(After Harrison)

Study first the normal development of the forelimbs in *Ambystoma* (Harrison 1918) In stage H28 and earlier notice the pronephros swelling immediately beneath somites 3–5 and a short distance posterior to the gill swelling The prospective forelimb area is represented by ectodermal and mesodermal material immediately ventral to the pronephros, including the ventral slope of the pronephric bud In stage H36 the limb bud becomes visible as a prominence separate from the pronephros In stage H37 it is a distinct bud which points in caudal direction It is at first cone shaped and then flattens at its distal end In stage H41 an indentation at its distal outline marks the two digits 1 and 2 which grow out rapidly in the succeeding stages Digits 3 and 4 follow successively at the ulnar border At the same time the elbow joint becomes visible Note the posture of the limb At first its palmar side faces the flank Then it rotates forward in the shoulder joint so that the animal supports itself with the forelimbs when it is at rest The balancers, which performed this function previously disappear at the time when the digits touch the ground

Limb-bud transplantations were among the earliest embryonic transplantations The pioneer work was done by Braus (1904) and Harrison (1907) on anurans in connection with certain problems of nerve outgrowth Later on Harrison introduced *Ambystoma* embryos as an unusually favorable object for limb transplantation since then very extensive experimental work has been done by Harrison and his students and by many other investigators using the limb bud as an object for the analysis of fundamental problems of determination (reviews in Mangold 1929 Swett 1937)

Detwiler (1933) has shown that the prospective limb material is determined as early as in the late yolk plug stage For our purpose it is advisable to use older stages preferably stages H25–H31 Harrison (1918) has demonstrated that the limb-forming potencies reside in the mesoderm and not in the epidermis of the primordium Removal of ectoderm does not interfere with limb development, whereas extirpation of the entire limb mesoderm, keeping the ectoderm intact in position usually results in the lack of a limb Also heterotopically transplanted limb mesoderm without ectoderm will give rise to a limb It is therefore essential for the success of the following experiments to include the mesoderm in the graft as completely as possible

Occasionally the transplant will form *duplicated limbs* The duplication may affect only the digits or it may affect the whole limb or any intermediate degree of duplication may occur In such cases the double limbs are mirror images of each other These duplications are of interest in several respects First they prove for an organ primordium what constriction and inversion experiments (pp 69-75) have proved for the whole egg in earlier stages that at the time of transplantation the individual skeletal muscle, and other elements were not rigidly determined in a mosaic like fashion (see p 94) Second the mirror imaging of all duplications shows that once a limb bud segregates into two there must be a reversal of the symmetry relations of one of the partners under the influence of the other partner The same mirror imaging is frequently found in the viscera heart, etc. of naturally occurring and artificially produced double monsters where it is known as *situs inversus* (see p 70) The problem of symmetry and the causes of symmetry inversion in duplicated limbs are discussed by Harrison (1921)

It is of interest to raise the donor embryos of limb transplantations and to find out if the extirpated primordium will be regenerated or not If the donor embryo shows no limb defect and the transplant is entirely resorbed one may suspect that not the limb primordium proper but adjacent tissue has been transplanted by mistake.

Experiment 15

Material

Amblystoma (any species) or *Triturus* stages H25–H31
standard equipment (p 41)
0 1 per cent solution of Nile blue sulphate

Procedure

1 Prepare at least a dozen embryos of stages H25–H31 preferably stages H28-H30 Select very healthy embryos. Remove all membranes

including the vitelline membrane, and transfer the embryos in a sterile pipette to sterile $\frac{1}{4}$ Holtfreter solution Older embryos which begin to show movements must be narcotized in chloretone or MS 222 during the operation (see p 40)

2 It is advisable to vital stain the donor embryos *in toto* by placing them for an hour in a 0 1 per cent solution of Nile blue sulphate

3 Transfer 2 embryos into the operation dish and, with the glass rod with ball tip mold two grooves, side by side into which the embryos will

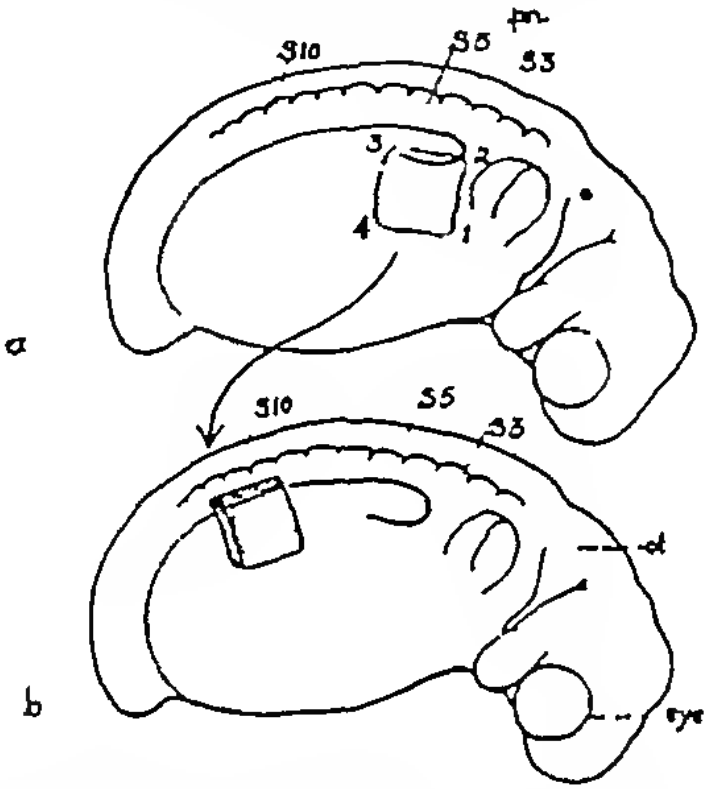

FIG. 22 —Transplantation of a forelimb primordium. *a* = donor embryo *b* = host embryo 1 2 3 4 = limb area. *ot* = otocyst *pn* = pronephros *S3* etc. = somite 3, etc.

fit when lying right side up Smooth the edges of the grooves very carefully Place the embryos in the grooves Have several glass bridges ready, slip them over the embryo and make sure that they have the proper size and bend

4 Prepare the host embryo All transplants should be made to the flank at some distance posterior to the pronephros swelling of the host, and approximately below somites 8-10 The position of the graft should be in exactly the same level as the host limb I e , immediately below the somites Transplants grafted to a more ventral position that is into the yolk do not take well and are frequently resorbed or extruded

With the glass needle cut a hole 2-3 somites in length into the ectoderm adjacent to and just beneath somites 8-10 Follow the technique as

m Experiment 14 under section 4 (p 83) Remove carefully a consider able amount of mesoderm cells with the tip of the glass needle so that a rather deep hole is laid open (Fig 22 *b*)

5 Extirpate the prospective limb area of the donor (Fig 22 *a*) Locate the pronephros swelling The limb area extends from the third to the fifth somites immediately below the pronephros swelling and includes the ventral part of the latter With the glass needle make a stitch through ectoderm and mesoderm in the groove between gill and pronephros swelling and stroke the hair loop against the needle until a cut is made (line 1–2 in Fig 22 *a*) Make a second cut parallel to the first one behind the pronephros using the sixth somite as a landmark (line 3–4) Then make two horizontal cuts one across the middle of the pronephric swelling (line 2–3 pronephric tubules may be exposed) and the other one parallel to the ventral midline of the embryo (line 1–4) Next with the tip of the needle lift out the entire cut area including as much of the deep mesoderm as possible Be sure not to lose too many mesoderm cells during the following transfer Work as fast as possible.

Note—Harrison and his students use the iridectomy scissors for extirpations Operations with this instrument are probably easier but iridectomy scissors are expensive and therefore usually not available for class use

6 For implantation transfer the transplant on the tip of the needle to the hole in the host proceed with the greatest care and try not to lose the orientation of the transplant If necessary drop the transplant near the host without changing its orientation and enlarge the hole Implant the graft in normal orientation press it into the hole with the glass needle or the hair loop and cover it quickly with a glass bridge Press the bridge firmly against the transplant push the edges of the bridge into the Permoplast.

7 Give the embryo a protocol number make a sketch and take a careful protocol including data on the amount of mesoderm transplanted and the orientation of the graft.

8 After a half hour remove the glass bridge very cautiously If the transplant has not healed in properly place the bridge back for another half hour The danger of damaging the embryo by prolonged pressure is much less than the chance of losing an improperly healed transplant, which is usually extruded

9 Transfer the embryo in a wide mouthed pipette to a Lily dish (label it) clean the wound of the donor embryo of adhering cells and transfer it to the same or to another dish.

Note—Make 3 or 4 operations of the same kind

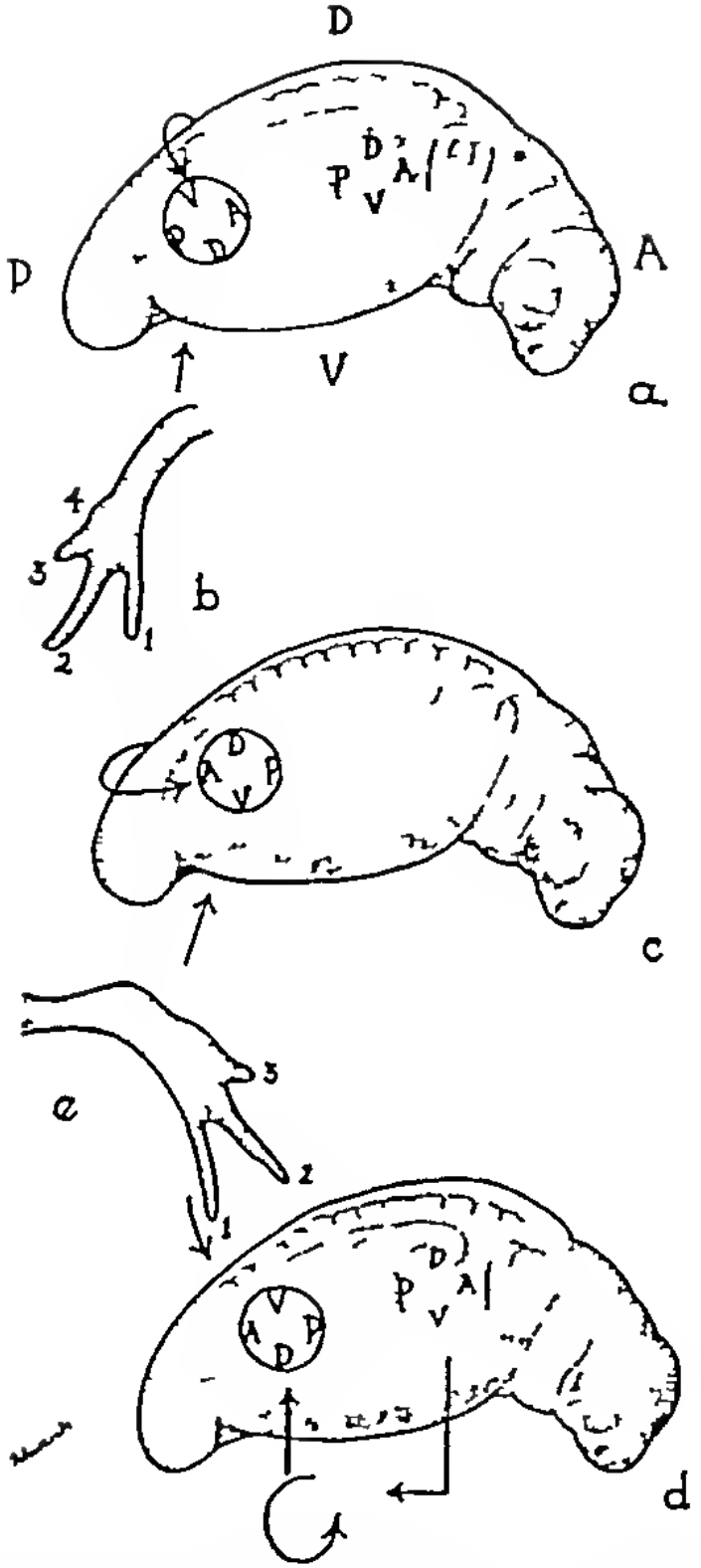

FIG 23 —Limb transplantations with inversion of axes *a* = transplantation of a left forelimb primordium to the right flank, with inversion of the *dv* axis (*ada*-orientation) *A* = anterior *P* = posterior *D* = dorsal *V* = ventral. The dotted circle and the letters in it indicate the position and axes of the host forelimb the solid circle and the letters in it indicate the transplant and its axes *b* = the transplant, resulting from this operation *c* = transplantation of a left forelimb primordium to the right flank with inversion of the *ap* axis (*apdd*-orientation); *d* = transplantation of a right forelimb primordium to the right flank, after rotation of 180° (*apdv*-orientation) *e* = the transplant, resulting from operations *c* and *d* (modified after S ett, 1937)

10 During the following weeks observe both host and donor frequently Narcotize both and study them in the narcotic. Take careful protocols and make sketches of all changes which you observe on the transplant and on the limb region of the donor Compare the development of the transplant with that of the host limb and with the left forelimb of the donor Watch carefully for a possible duplication of the transplant try to detect and sketch it at its inception This will help in the later interpretation of the symmetry relations (see Harrison, 1921)

d) LIMB TRANSPLANTATIONS COMBINED WITH INVERSION OF AXES

The origin of polarization and of symmetry relations in an organism or in an organ is one of the central problems of experimental embryology Harrison (1921) in a classical study analyzed this problem by transplanting limb primordia in such a way that their axes would not coincide with those of the host embryo In a limb primordium Harrison distinguishes 3 axes the anterior posterior (*a–p*) axis, the dorsoventral (*d–v*) axis and the mediolateral (*m–l*) axis which coincide with the corresponding axes of the embryo Left primordia were transplanted to the right flank (Fig 23 *a* and *c*) or right primordia were rotated 180 but kept on the same side (Fig 23 *d*) Transplantations from one side to the other can be done in two ways either by moving the transplant over the back, so to speak (Fig 23 *a*)—in which case the *dv*-axis is inverted with respect to the host but the *ap*-axes of the host and transplant coincide (*aadv*-orientation in Harrison's terminology)—or by shifting the transplant around the tail so to speak (Fig 23 *c*)—in which case the *ap*-axis is reversed but the *dv*-axis is not (*apdd*-orientation) Rotation by 180 on the same side results in an *apdv*-orientation Briefly the results were as follows The *ap*-axis is irreversibly fixed In all instances a transplant in *ap*-orientation will grow forward instead of caudad (Fig 23 *e*) It will follow its original trend uninfluenced by adjacent tissues The *dv*-axis however is not irreversibly fixed at least not up to stage H32 (in *A punctatum*) Transplants in *dv*-orientation behave as if they were in a *dd*-orientation with the first digit growing outward It is to be concluded that the *dv*-axis becomes polarized by influences from adjacent host tissue This explains the startling phenomenon that, in certain combinations a left primordium will give rise to a right limb (for instance in operation Fig 23 *a b*) or a right primordium can be made to form a left limb even if it stays on the right flank (Fig 23 *d e*) From stage H35 on the *dv*-axis is also irreversibly fixed Stages H33 and H34 are transitional stages For all details see Harrison (1921) More recent studies are reviewed in Swett (1937) The experiments clearly illustrate a point which was emphasized on page 80

viz. a primordium may self-differentiate with respect to one characteristic and at the same time, be dependent on extrinsic factors in other respects.

Experiment 16a. Reversal of the *dv*-Axis

Material

Ambystoma not older than stage H31
standard equipment

Procedure

In order to facilitate the orientation of the transplant vital-stain the anterior border of the *left* forelimb primordium Operate as in Experiment 15 Implant to the right flank Orient the transplant as in Figure 23 *a* Fit the anterior (marked) border of the transplant to the anterior border of the implantation groove in the host. Note the direction of out growth

Experiment 16b. Reversal of the *ap*-Axis

Procedure as before but fit the anterior (vital stained) border of the transplant to the posterior border of the implantation groove (Fig 23, *c*) Do you expect a result different from Experiment 16*a*?

Experiment 16c Rotation 180

Proceed as indicated in Figure 23 *d* What do you expect? How will the results compare with those of the preceding experiments?

Experiment 16*d* Reversal of the *dv*-Axis in Older Stages

Repeat Experiments 16*a* or 16*c* in stages older than H35 How does the outcome compare with that of Experiments 16*a* and 16*c*?

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e) Transplantation of Gill Primordia
(After Severinghaus)

The external gills of salamander larvae consist of three branches with secondary filaments They are fully developed in stage H41 In the stage

series (Fig 45) trace them to earlier stages In tail bud stages as early as stage H25 or stage H26 they are clearly distinguishable as lateral swellings on the head slightly ventral and posterior to the optic vesicle Familiarize yourself with the normal development of gills

Harrison (1921) has shown that the gills of *A maculatum* are determined as early as in stage H21 All three germ layers contribute to the formation of the gills The respective role of each one of them in the determination of the size and shape of gills has been investigated by means of heterotopic, beteroplastic and xenoplastic* transplantation of the three components separately The considerable literature on this subject is discussed in Severinghaus (1930) and Rotmann (1935) The following experiments will demonstrate merely that the three layered gill swelling is self differentiating in its gross morphology in early tail bud stages, that is long before visible differentiation takes place

EXPERIMENT 17

Material

Amblystoma any species stages H26–H29
standard equipment (p 41)

Procedure

1 Select two embryos of approximately the same stage Remove all membranes Place them side by side in two grooves in the operating dish (as in Expt 14 under secs 1–3 [p 83]) right side up Operate in full strength Holtfreter solution

2 *Prepare the host embryo* —With glass needle and hair loop prepare a rather large groove in the flank of the host embryo Choose one of the following sites posterior or ventral to anterior limb bud immediately ventral to somites 9–12 in the place of the eye For this and the following procedure follow the technique described in Experiment 14, under sections 4–8 (p 83)

3 *Extirpation of the gill primordium* —Cut out the larger part of the gill swelling using glass needle and hair loop Cut very deeply so that the pharyngeal cavity is exposed Lift out the block of tissue and transfer it cautiously to the site of implantation using the tip of the glass needle or the hair loop

4 Enlarge the hole if necessary Fit the transplant in and hold it in position with a glass bridge for 30 minutes or longer

5 After the transplant is healed in lift the glass bridge cautiously

Heterotopic = transplantation to a different position heteroplastic = transplantation between embryos belonging to different species xenoplastic = transplantation between embryos belonging to different genera, families, or more distant taxonomic categories

clean the edges of the wound and transfer the embryo to a dish filled with $\frac{1}{10}$ Holtfreter solution

6 Take a protocol, note the orientation of the transplant. Give the embryo a serial number Operate 3–5 embryos in the same way In some of them orient the transplant in inverted position.

7 On the following day observe and protocol all changes in the transplant Compare its development with that of the host gills. In what orientation do the transplanted gills grow out? Are they of normal size and shape? Are they vascularized? They will be resorbed eventually

Further suggestions —Study the papers of Harrison Severinghaus, and Rotmann and repeat some of the experiments in which ectoderm alone or mesoderm alone was transplanted or rotated

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5 REGULATIVE PROPERTIES OF ORGAN PRIMORDIA (MORPHOGENETIC FIELDS)

a) INTRODUCTORY REMARKS TERMINOLOGY

In the neurula stage many regions of the amphibian embryo have acquired a considerable degree of self-differentiating capacity This holds for the limb-forming area, the eye forming area, for the nose, ear, heart, balancer region etc. These areas have very peculiar properties First of all they show a high regulative power When part of a limb eye, or heart primordium etc. is transplanted the transplant will tend to form a whole structure as will the fragment which was left behind in the donor In this respect the organ primordia resemble the egg in the 2 or 4-cell stage They are not composed of a mosaic pattern of smaller self-differentiating and specialized units On the contrary each part must contain within itself a full complement of all factors which are necessary for the formation of a whole Driesch called such systems ' harmonious equipotential systems. If any given part of such a system is potentially capable of forming the whole organ yet only one proportionate limb eye or heart develops eventually then rigid restrictions of potencies must be imposed on the parts They are assigned to limited specific tasks within the framework of a whole and mutual adjustments between the parts must take place At a time when the limb area as a whole is self

differentiating as is shown by transplantation experiments (p 86) the finer structural details within this area, such as individual skeletal elements muscles etc. are not yet determined they gradually become established in later stages by mutual interactions of the parts and possibly through other mechanisms Harmonious equipotential systems illustrate the epigenetic nature of development, as well as the relativity of the terms self-differentiation and "determination (see p 80)

The organ forming areas which are thus blocked out in the rough have at first no distinct boundaries. The capacity for limb or eye formation may extend beyond the cell area which, in normal development, will actually form the limb or the eye This was demonstrated by experiments in which the entire prospective limb- or eye forming area was extirpated yet a limb or an eye was formed by adjacent cells which closed the wound Finally within each primordium there seems to exist a gradient of organ forming capacity with a peak in the center of the field and a gradual decline toward the periphery Embryonic areas which exhibit the following four characteristics are called 'morphogenetic fields (1) They are *self differentiating* systems, as shown by heterotopic transplantation (2) they are *regulative* systems as shown by the formation of normal organs after removal or transplantation of half primordia or after superimposition of two whole primordia (3) the specific organ forming *potencies extend beyond the borders* of the prospective organ forming areas and (4) these self differentiating regulative areas are *gradient fields* For further discussions of the field concept see the books by Huxley and De Beer, by Spemann and by Weiss

In the following experiments the properties of morphogenetic fields will be illustrated by experiments on the limb the heart, and the eye

b) EXTIRPATION EXPERIMENTS ON THE LIMB FIELD
(After Harrison 1918)

The first extensive analysis of field properties was made on the forelimb of *Ambystoma* by Harrison (1918) In tail bnd stages the limb area is a disk immediately ventral to the pronephros extending from the anterior border of the third to the posterior border of the fifth somite Its dorsal most part covers the ventral part of the pronephros (Fig 22 *a*) The limb-forming potencies reside in the mesodermal cells of this area.

Harrison first made a systematic potency test of half-disks anterior posterior dorsal, and ventral halves were extirpated It was found that any half-disk is capable of forming a whole limb although the percentage of normal limbs resulting from the operation varied considerably Detwiler (1918) supplemented these experiments by heterotopic transplantation of

dorsal or of ventral half-disks, both of which gave rise to normal limbs. The limb area has thus beeo shown to be a "self-differeotiating harmoni ous equipotential system as the title of Harrison s paper indicates. Next the enture limb disk was removed Normal limbs developed from cells which migrated into the wouod from the periphery When the diameter of the extirpated disk was increased from 3 to 4 and 4½ somites, oormal limbs were still formed in a certain perceotage of cases. When the amount of extirpated tissue was further increased, no regeneration took place The percentage of "regenerating limbs was lower when larger disks were removed or when the wound was thoroughiy cleaned of mesoderm cells Thus It is shown that the limb-forming potencies extend beyond the area which actually enters into limb formation (i e , the prospective limb area) aod that they are higher in the center than at the periphery

The student should consult the paper of Harrison (1918) for all details.

Material for Experiments 18–21

Amblystoma any stage between H24 and H30
standard equipment (p 41)

Experimental procedure for Experiments 18–21

Operate in full streogth Holtfreter solution Operate always on the right side of the embryo Narcotize in chloretone 1 3,000 or MS 222 1 3 000 if necessary Proceed as follows

1 Choose a number of healthy tail bud stages Remove all membranes. Wash in sterile Holtfreter solution

2 Transfer ooe embryo to the operation dish Prepare a groove of ade quate size into which the embryo fits right side up Locate the pronephros swelling and the limb disk (Fig 22, *a*)

3 With the glass oeedle (or the iridectomy scissors) cut out the desired part of the limb area make a rectangular hole if you use the glass needle

4 Make a careful sketch prepare a protocol label the embryo and transfer it to a Lily dish cootaining ⅓ Holtfreter solution

5 Observe the healing of the wound in the following hours and the differentiatioo of the limb in the following weeks Make sketches and take careful protocols Does the regenerating limb catch up with the left (control) limb?

EXPERIMENT 18

Removal of the dorsal half of the limb area —Cut out the dorsal half of the square 1 2 3 4 in Figure 22 *a* Make 3–5 operations *In some cases* clean the wound carefully of all mesoderm cells with the tip of the glass

needle in other cases leave some mesoderm behind. Protocol in each instance the amount of mesoderm left in the wound Note differences in the regulation

EXPERIMENT 19

Removal of the anterior half of the square 1 2 3 4 in Figure 22 *a*

EXPERIMENT 20

Removal of the entire prospective limb area 3 somites in diameter (1–2–3–4 in Fig 22, *a*)

EXPERIMENT 21

Removal of an area 4 somites in diameter centering ventrally to the fourth somite

Note—In evaluating the results one must bear in mind that not all quadrants of the limb disk share equally in the formation of the limb itself For instance the ventral half contributes less than does the dorsal half etc. The prospective significance of the different sectors has been worked out by Swett (1923) using the vital staining method

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SWETT F H. 1923. The prospective significance of the cells contained in the four quadrants of the primitive limb disc in *Amblystoma* Jour Exper Zool 37 207

c) FORMATION OF TWO HEARTS

The amphibian heart originates from two separate lateral primordia the free ventral edges of the left and right hypomere which fuse eventually in the mid ventral line According to Copenhaver (1926) in *A maculatum* stage H22 the two primordia are a considerable distance apart from each other they reach the mid ventral line in stage H27 The outline of the right heart primordium at this stage is indicated in Figure 24 *c* By stage H33 the endocardial tube is formed but not yet curved The formation of the different subdivisions of the heart by differential growth occurs in the succeeding stages Circulation begins at about stages H36–H37 the first spontaneous pulsations begin somewhat earlier The student should review details of heart development in a textbook of embryology and should read Copenhaver (1926 1939) before starting the experimental work.

The *field properties* of the heart forming area and of the surrounding tissue have been demonstrated by the same potency tests which were applied to the limb

1 *Relative self-differentiation* —Heterotopic transplantation of the heart primordium at stage H28 results in the formation of a fairly well-differentiated pulsating heart (Copenhaver, 1926) Such hearts may show typical subdivisions, curvature, etc. yet they are never completely normal, although they may be incorporated in the blood stream of the host. As Stoehr (1925, 1929) has pointed out, certain structural differentiations are dependent, even in late stages on extrinsic factors (normal blood transfusion normal spatial relations to adjacent structures such as the liver etc.) This point illustrates again the relativity of the concept of "self-differentiation'

2 *Regulative properties* —These have been demonstrated in the following way If one interferes with the union of the two lateral primordia by implanting foreign tissue mid ventrally between them, each of the primordia will form a whole heart (Ekman, 1925 Copenhaver, 1926) Such double hearts usually pulsate with different rates They are usually mirror images of each other, the left heart being normal, the right heart showing an inversion of its symmetry (situs inversus cordis, see Fig 24 *d*) Exactly the same type of inversion may be found in identical twins of *Triton* produced by constriction (p 70) Regulations may also be demonstrated by *extirpation of parts* of the primordium (Copenhaver, 1926) Anterior posterior, left, and right halves will regulate to form more or less typical whole structures Such hearts from fragments of the primordium approach normal tubes with typical subdivisions they are definitely not half-structures This experiment can be done successfully even at a stage when the heart tube is already formed, i e in stage H33 of *Ambystoma*

3 *The heart field extends beyond the limits of the prospective heart-forming area* —The complete extirpation of the latter in *Ambystoma* is usually followed by the regeneration of either one or two hearts Even the extirpation of an area exceeding considerably the heart forming area proper may be followed by the formation of small single or double hearts (Copenhaver 1926)

The following experiments illustrate points 2 and 3

EXPERIMENT 22 BLOCKING OF THE FUSION OF LEFT AND RIGHT PRIMORDIA

(After Ekman, 1925 and Copenhaver, 1926)

Material

Rana or *Ambystoma* embryos (*For hosts* use medullary-plate or medullary fold stages *for donors* use late neurulae [closing folds] or early tail-bud stages)

standard equipment (p 41)

Procedure

1 Take donor and host out of all membranes and place them side by side in grooves in an operating dish. Orient the donor right side up and the host ventral side up Operate in full strength Holtfreter solution.

2 With the glass needle make a longitudinal slit in the median ventral line of the head of the host. The slit should extend from the outer edge of the medullary fold backward to about one third of the entire length of the embryo Cut deeply and make a wide gap without injuring the heart mesoderm to the left and the right (Fig 24 *b*)

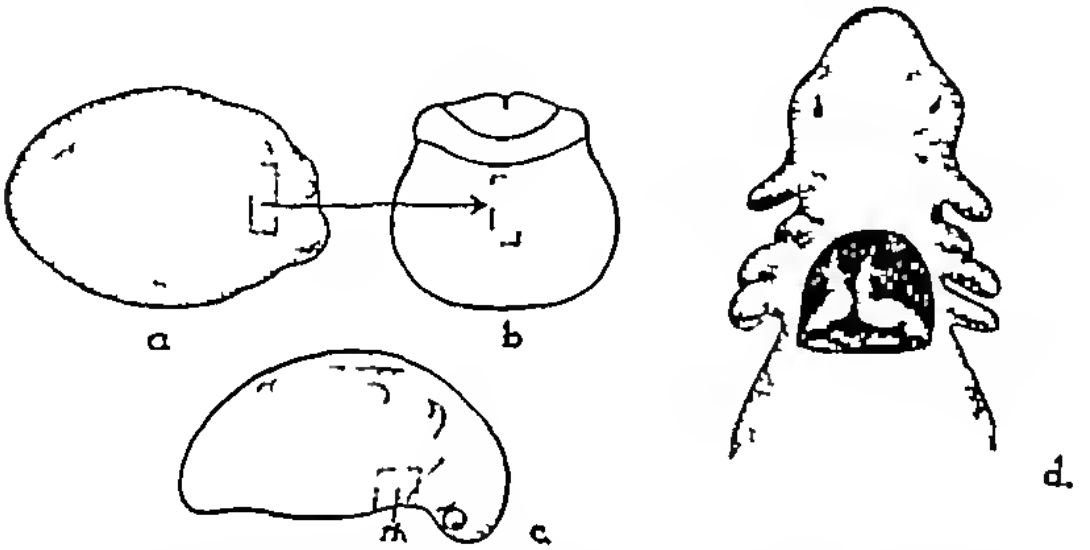

FIG. 24.—Production of two hearts out of one primordium (*c d* after Copenhaver 1936) *a*=donor embryo the dotted lines indicate the strip of tissue (gill area) which is implanted in the region indicated in *b* by dotted lines *b*=host embryo *d*=heart region of host embryo dissected (note that the two hearts are mirror images of each other) *c*=the right half of the prospective heart area (*rh*) in stage 17

3 The tissue which is to serve as a block must be self-differentiating or else it might be incorporated in the host heart Therefore slightly older donors are chosen The prospective gill region was found to be suitable as a block but any other tissue—for instance somites—will serve the same purpose With a glass needle cut out a long and narrow strip from the prospective gill area of the donor (Fig 24, *a*) The strip should include all three germ layers. Cut deeply until you reach the pharyngeal cavity Implant this strip in the slit of the host in a longitudinal direction Take care that the epidermis of the transplant is adjoining to that of the host. Place a glass bridge over the transplant for 30 minutes or longer

4 Remove the glass bridge after the transplant has healed in discard the donor place the host in another dish in $\frac{1}{4}$ Holtfreter solution Take a careful protocol. Rear it together with a few unoperated embryos of host age

Note—Make 3 or more operations

5 Allow the embryos to develop for 2–4 days Narcotize the host and a control place them in grooves in an operation dish, ventral side up. Observe pulsations through the epidermis If the epidermis is not sufficiently transparent dissect it away carefully with a strong glass needle iridectomy scissors or watchmaker forceps Carefully avoid damage to the beating hearts Note the heart beat. Are two hearts present? Do they beat *synchronously? Take notes Practice* the *dissection in vivo, on a* normal embryo first.

6 *Dissection*—Fix the host and several controls in 10 per cent formaldehyde + 5 per cent aqueous solution of nitric acid Dissect carefully with a glass needle Dissect a control embryo first identify the parts of the heart. Then dissect the operated animals, make sketches, and take a protocol You will find partial duplications if the block did not extend sufficiently far anterior or posterior If two hearts are present, study their symmetry relations (Fig 24, *d*)

d) PARTIAL REMOVAL OF THE PROSPECTIVE HEART REGION

EXPERIMENT 23

(After Copenhaver, 1926)

Material

Amblystoma stages H26–H28
standard equipment (p 41)

Procedure

1 Remove all membranes. Place the embryo in a groove in the operation dish ventral side up

2 With a glass needle remove the right half of the heart area as indicated in Figure 24, *c* Apply the technique described in Experiment 14 under sections 4–8 (pp 83–84) Extirpate ectoderm and mesoderm Do not cover the wound Make several operations

3 Transfer the embryos to Lily dishes ($\frac{1}{1}$ Holtfreter solution) and allow them to develop for several days Notice the heart beat

4 Fix and dissect the embryos from the ventral side with a strong glass needle as in Experiment 23 Take protocols make sketches.

e) REMOVAL OF THE ENTIRE PROSPECTIVE HEART REGION

EXPERIMENT 24

(After Copenhaver 1926)

Procedure

Proceed as in Experiment 23 Remove area *rh* (Fig 24 *c*) and the corresponding area on the left side

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f) EXTIRPATION OF PARTS OF THE EYE FORMING MATERIAL IN THE NEURULA STAGE

(After Adelmann)

The prospective eye forming area has been localized in the medullary plate stage by Manchot (1929) using the method of vital staining (see p 59 and Fig 17 *a*) The materials for the two eyes are located close together near the median region of the anterior medullary plate They are connected by a narrow median strip which represents the prospective chiasma region During the process of neurulation these primordia move apart, in a lateral direction and material which was originally located posteriorly moves forward and forms that part of the brain floor which eventually separates the eyes

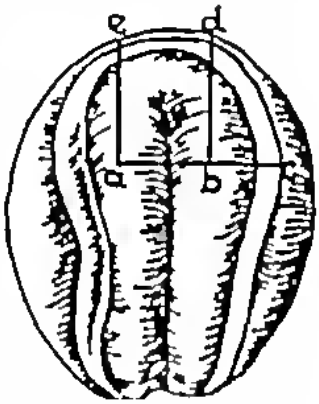

FIG. 25 — Extirpations in the eye field of the medullary-fold stage (see text)

The anterior part of the medullary plate represents an eye field in the sense defined above (p 95) It is self-differentiating and at the same time a harmonious equipotential system it shows a gradation of eye forming potencies and it extends beyond the boundaries of the actual prospective eye forming areas These conclusions are deduced primarily from the extensive series of transplantation and extirpation experiments on the anterior medullary plate by H B Adelmann (1929*a* 1929*b* 1930) The student is referred to Adelmann's review (1936) and to Mangold (1931)

If a median strip of the medullary plate (Fig 25 *a–b–d–e*) is transplanted to the flank of another embryo the donor may develop two complete eyes by regulation and the transplant may also form either 1 or 2 well formed eyes Thus the eye field may give rise to 3 or 4 eyes The removal of a lateral area *b–c–d* may likewise be followed by complete regulation In order

to suppress the formation of 1 eye entirely, it is necessary to make a defect which extends to the median line and in posterior direction as far as the level of the broadest portion of the neural plate Thus shows that the eye forming potencies extend beyond the prospective eye forming area A mediolateral gradient of eye forming potencies was established by comparing the percentages of eye formation from lateral and from median strips It was as high as 70 per cent for median strips and only 11 per cent for lateral strips under otherwise identical conditions If the "peak ' of eye forming capacity is in the center of the medullary plate why are 2 lateral eyes instead of 1 single median eye formed in normal development? Adelmann (1930) has shown that the underlying entomesoderm is responsible for this effect It creates bilaterality by "reinforcing ' the eye potencies in lateral regions When median strips of the medullary plate were transplanted with and without the entomesodermal substrate (prechordal mesoderm) those with substrate formed 2 eyes in almost 50 per cent of the cases, while those without substrate never formed 2 whole eyes and at best only a single eye The explanation of the origin of 1-eyed monsters (cyclopia) is along these lines The presence or absence of the substrate will have to be given special consideration in the following experiments

Material for Experiments 25–29

Amblystoma, any species stages H15–H18
standard equipment (p 41)

EXPERIMENT 25 REMOVAL OF THE MEDIAN ONE THIRD OF THE MEDULLARY PLATE WITH SUBSTRATE (*a–b–d–e* FIG. 25)

Procedure

1 Select a number of healthy embryos Remove all membranes

2 In an operation dish prepare a groove of proper size and place an embryo in it. Operate in full strength Holtfreter solution

3 With the glass needle and hair loop cut out a block of tissue along the lines *a–b–d–e* Follow the technique described in Experiment 14 under section 4 (p 83) Cut deeply until the archenteron is exposed Remove the medullary material and its substrate

4 Make a protocol sketch Allow the wound to heal in full-strength Holtfreter solution for 1–2 hours

5 Make several operations of this type Place them all in a dish in ⅓ Holtfreter solution

6 After 24 hours take a protocol of the condition of the wound Discard all disintegrating embryos

7 During the following days observe the development of the eyes

8 When the embryos have reached stages H36–H40 fix the entire material in 10 per cent formaldehyde. Dissect the head skin away Compare the size of normal and regulated eyes.

Experiment 26 Removal of the Median Strip without Substrate (*a–b–d–e* Fig. 25)

Proceed as in Experiment 25 but carefully avoid injury to the prechordal entomesoderm. Scrape off the ectoderm with the tip of the glass needle and the hair loop

Experiment 27 Removal of the Lateral One third with Substrate (*b–c–d* Fig. 25)

Proceed as in Experiment 25 Make first cut *b–d*, then *b–c* then *c–d*

Experiment 28 Removal of the Lateral One third without Substrate (*b–c–d* Fig 25)

Proceed as in Experiments 25 and 27 Carefully separate medullary plate from substrate using hair loop and glass needle

Experiment 29 Removal of Two-thirds of the Medullary Plate without Substrate (*a–c–d–e* Fig. 25)

Proceed as before. Make the cuts in the following sequence *a–e* *a–c*, *c–e*

Make several operations of each type Compare your results with those of Adelmann

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g) PARTIAL AND TOTAL EXTIRPATION OF THE OPTIC VESICLE

In the early tail bud stages the optic primordium has proceeded to form the optic vesicle The prospective significance of the different parts of the vesicle—retina pigment epithelium, stalk—has been mapped by Petersen (1923, see Fig 26, *b*)

The optic vesicle is still capable of regulation, both in urodeles and in anurans Even small fragments of the vesicle may form normal, though smaller eyes (review in Mangold 1931) The prospective retina may replace the prospective pigment epithelium if the latter is removed and vice versa (Dragomirow, 1932, 1933, 1935) The optic vesicle still has field properties These findings illustrate clearly a point of theoretical importance (p 80) The optic primordium is self-differentiating as a whole but its parts (retina etc.) are not yet self-differentiating units, even in relatively late stages Determination is a process which continues over a considerable period, during which first the general and then the detailed characters of an organ primordium become irreversibly fixed The optic vesicle differs from other morphogenetic fields in one respect eye-forming properties do not transcend the boundaries of the prospective eye area No eye regeneration occurs when the optic vesicle is entirely extirpated This result again illustrates a general principle to be emphasized further in the chapter on "Regeneration regulation and regeneration are properties of fields and not of the organism as a whole A fragment of a morphogenetic field is capable of restoring the lost parts, but the organism is not capable of restoring a field once it is lost entirely

Experiment 30 Removal of the Distal Region of the Optic Vesicle

Material

Rana sylvatica, palustris, stages PM16–PM17 (*R pipiens* embryos are sticky in these stages and therefore less desirable They may be used if no other material is available)

standard equipment (p 41)

Procedure

The student is advised to dissect the eyes of normal embryos (fresh or fixed in 10 per cent formaldehyde) before the operations are started

1 Select a number of healthy embryos remove all membranes Place one embryo in a groove in the operation dish, right side up Operate in full strength Holtfreter solution

2 Locate the right optic vesicle it forms a slight bulge on the surface With the glass needle and hair loop cut out a square piece of epidermis

over the eye (*l e* in Fig 26 *a*) Remove the skin The window should be so large that the optic vesicle (*o.v*) is clearly exposed

3 Remove the outer (distal) half of the optic vesicle with the glass needle (cut *a–b* in Fig 26 *b*) Make sure under the high power that the cavity of the vesicle is exposed Leave the embryo in full strength Holtfreter solution until wound healing is well under way—for 1–2 hours (It is not necessary to cover the wound with foreign epidermis Adjacent epidermis will grow over)

4. Transfer the embryo to a dish with $\frac{1}{3}$ Holtfreter solution Take a protocol.

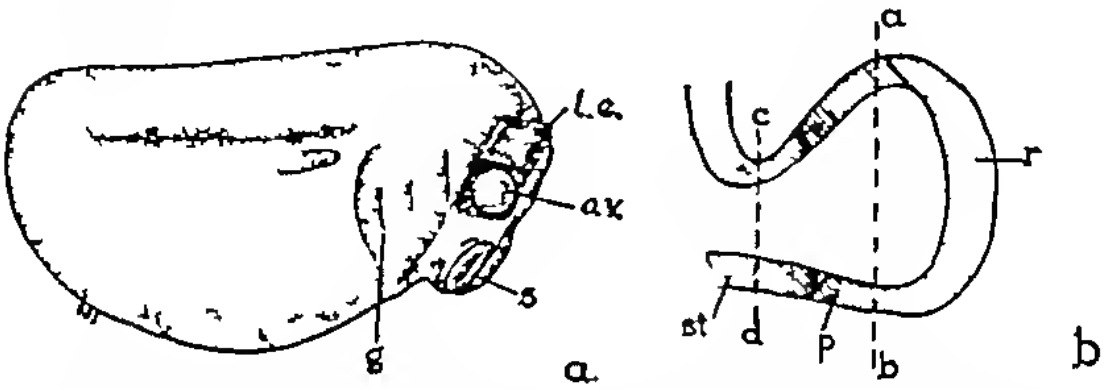

Fig 26—Eye extirpations in the tail bud stage *o*=the optic vesicle exposed *g*=gill region *l e*=lens epithelium *o v*=optic vesicle *s*=suckers *b*=the prospective areas of the optic vesicle (from Mangold, 1931 after Petersen) *p*=pigment epithelium *r*=retina *st*=optic stalk.

5 When the cornea over the eyes has become transparent (1 week or longer) fix the embryo in 10 per cent formaldehyde and dissect the skin over the eye Note the degree of regeneration the size, etc. of the operated eye in comparison with the left (control) eye Make sketches

Make several operations

Experiment 31 Total Extirpation of the Optic Vesicle

Material and procedure as in Experiment 30 except that the entire vesicle is removed by a cut near the brain (*c–d* in Fig 26 *b*) Make certain under the high power that only the narrow opening of the stalk into the brain rather than the wide lumen of the optic vesicle remains Fix and dissect the embryo after 1 week Notice the complete absence of the eye

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6. EMBRYONIC INDUCTION

a) INTRODUCTORY REMARKS TERMINOLOGY

The experiments to be discussed next center around the following problems What factors are instrumental in the process of determination? How is the determination of the medullary plate of the eye, of the balancer, accomplished? There is no one answer to these questions. A multitude of different agents are at work in each instance and each primordium requires a special analysis

Of the few general mechanisms so far discovered, that of "embryonic Induction is of considerable importance It may be defined as a process in which one embryonic area, the *inductor*, calls forth a specific differentiation in an adjacent embryonic tissue by contact The two best analyzed and most widely known cases are the lens induction by the optic vesicle and the induction of the medullary plate by the underlying mesoderm

The term ' induction includes apparently, quite heterogeneous types of interactions ranging from mere "trigger actions to highly specific interactions Induction undoubtedly does not comprise a physiologically uniform group of phenomena. If one bears this in mind then the use of this convenient term as defined above will do no harm

Embryonic induction can be demonstrated in two ways by extirpation and by transplantation If, after the extirpation of an embryonic area an adjacent organ fails to differentiate, then a causal interaction of the inductor type suggests itself However, crucial evidence for induction can be obtained only from transplantation experiments because they give a positive rather than a negative indication Transplantations as proofs for induction are always of the following type the structure which is suspected of being an inductor is combined with relatively indifferent (that is not self-differentiating) tissue from a remote part of the same or of another embryo or even from an embryo of another species For instance the optic vesicle is combined with belly ectoderm or the archenteron roof of one species is made to interact with ventral gastrula ectoderm of another species If under these circumstances a lens or a neural tube respectively appears in structures which would never have formed these

structures by themselves then the inductor capacities of eye or of archenteron roof are positively established. Both of these examples will be treated in the following experiments. The student is referred to the chapters on induction in the books of Huxley and De Beer, of Spemann and of Weiss

b) FAILURE OF LENS FORMATION AFTER EXTIRPATION OF THE EYE PRIMORDIUM IN THE MEDULLARY PLATE

(After Spemann)

The optic cup and the lens originate from two different sources the former as an evagination of the forebrain, the latter as an invagination of the head ectoderm The fact that the lens is formed at the point where the optic vesicle makes contact with the overlying ectoderm is suggestive of a causal relation between the two Spemann was the first to test this assumption experimentally He extirpated the primordium of the optic vesicle and found that no lens would differentiate although the prospective lens area was left undisturbed It was concluded that the latter requires an inductive stimulus from the optic vesicle for lens formation His first experiments were made on the European grass frog *R. temporaria* When another species *R esculenta* was used a lens though small and incomplete was formed even in the absence of the optic vesicle These experiments were extended to other species and repeated by other investigators. Spemann s conclusions were confirmed first the optic cup is instrumental in the formation of the lens second the dependence of the lens epithelium on the optic cup differs in different species. The lens has since become one of the classical objects for the analysis of embryonic induction (reviews in all textbooks see also Spemann 1938 and Mangold 1931)

Most of the American urodeles and anurans in which the extirpation of the optic primordium has been performed so far show a complete lack of lens differentiation in the absence of the optic vesicle This holds for *R. sylvatica R. palustris* (Lewis 1904 1907) *R catesbeiana* (Pasquini 1932) and *A maculatum* (Harrison 1920)

The extirpation of the eye primordium can be done either in the medullary plate stage or in the early tail bud stage before a contact between optic vesicle and epidermis is established Experimentation on the earlier stage has the advantage that the lens-forming area need not be disturbed at all whereas in the tail bud stage the lens epidermis must be lifted to get access to the eye and then healed back again

EXPERIMENT 32

Material

Rana sylvatica (stage PM14) or *R pipiens* (stage Sh14) or corresponding stages of *R palustris* or *calesbeiana* or *Ambystoma* stage H15 or stage H16

standard equipment (p 41)

Procedure

The aim of this experiment is to remove the eye primordium (part of anterior medullary plate) without disturbing the lens-forming area, which is located just outside the medullary plate or fold (see Fig 17, *L*) The experiment is identical with Experiment 28 (p 103), except that a median cut should be made instead of *b–d* (Fig 25), in order to prevent eye regeneration Again, first make the median cut then the cut in direction *b–c* and finally the cut in direction *c–d* Carefully avoid any damage to the mesodermal substrate and to the prospective lens area. Follow the technique described for Experiments 25 and 26 (pp 102–3) Operate in full strength Holtfreter solution and allow wound healing in this solution for several hours Take protocols of the operation

Operate on several embryos

Fix the larvae in 10 per cent formaldehyde when the swimming stage is reached and the cornea over the left eye is clear Note the opaque left lens and the absence of a transparent cornea on the right side Carefully dissect the skin of the right side of the head Find out if the right eye is completely absent. The presence of a small eye may be due to an incomplete extirpation and a regulation of the fragment. Note if a small, whitish opaque lens is attached to such a regenerated eye The complete absence of all rudiments of a lens can be definitely established only by sectioning

c) FAILURE OF LENS FORMATION AFTER EXTIRPATION OF THE OPTIC VESICLE IN EARLY TAIL BUD STAGES

EXPERIMENT 33

Material

Rana sylvatica stage PM16 or stage PM17 or *R pipiens* stage Sh16 or corresponding stages of *R palustris* (*R sylvatica* and *palustris* are preferable)

standard equipment (p 41)

Procedure

Note the projecting optic vesicles inspect the head from all sides

1 Remove all membranes

2 Operate in full strength Holtfreter solution Place the embryo in a Permoplast groove, right side up With the glass needle cut out a square flap of epidermis over the eye, cutting on three sides only (Fig 26 *a*) Follow the technique described in Experiment 14 (p 82) Peel the epidermis off the optic vesicle carefully In early stages there is no close contact as yet between the two Reflect the flap of ectoderm Cut out the optic vesicle at its base using the glass needle as in Experiment 31 (p 105) Turn the flap back again flatten it out cautiously with the hair loop or glass needle and place a glass bridge over it. If it fits well it will be healed in 20–30 minutes Control the healing carefully if the flap shrivels then neighboring epidermis will grow over the wound The lens will be absent in either case but only the instance in which prospective lens epithelium proper fails to form a lens is conclusive for the present problem.

3 Prepare a protocol and transfer the embryo to a dish with $_1{}^1{}_7$ Holtfreter solution.

Operate several embryos in the same way

4 Fix the swimming larvae several days later in 10 per cent formaldehyde Proceed as in Experiment 32

d) LENS INDUCTION AFTER REMOVAL OF THE PROSPECTIVE LENS EPITHELIUM

Once a causal relation is established between optic vesicle and lens formation a number of questions arise concerning the nature of lens induction For instance is the optic vesicle capable of inducing a lens out of foreign epidermis? This can be tested either by transplanting foreign epidermis in the place of the prospective lens epidermis or simply by removing the lens epidermis and allowing adjacent head epidermis to grow over the eye

EXPERIMENT 34

Material

as in Experiment 33

Procedure

1 Remove all membranes

2 Operate in full strength Holtfreter solution Place the embryo in a Permoplast groove right side up With the glass needle remove a square piece of epidermis which covers the optic vesicle (*l e* in Fig 26 *a*) Carefully avoid any injury to the optic vesicle Leave the embryo in the full strength Holtfreter solution for 0 40 minutes Observe the growth of adjacent head epidermis over the wound

3 Take a protocol and transfer the embryo to a Lily dish containing ⅓ Holtfreter solution

4. After the embryo has reached the swimming stage fix it in 10 per cent formaldebyde After a few minutes the lenses will become visible as opaque white structures Note that the epidermis which has grown over the right eye has become transparent and forms a normal cornea. Dissect the cornea away from both eyes and study the size and shape of the right lens which has been induced from head epidermis

Further suggestion —Transplant the optic vesicle to the flank of an other embryo Make a deep groove ventral to the middle somites and fit the optic vesicle into it. Heal a flap of epidermis over the transplant or place a glass bridge over the eye to keep it in position and allow adjacent epidermis to grow over Again find the lens by dissection after fixation in 10 per cent formaldehyde

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c) THE ORGANIZER EXPERIMENT TRANSPLANTATION OF THE UPPER LIP OF THE BLASTOPORE

(After Spemann and H Mangold, 1924)

Note —Before starting this experiment read the chapter on organizers in any textbook Read the section on gastrulation (p 43)

The organizer experiment (Spemann and H Mangold 1924) represents the classical case of a complex induction The authors discovered that the upper lip of the blastopore of a urodele gastrula when transplanted to the flank or the ventral region of another embryo of the same stage would self-differentiate into mesodermal structures such as notochord and somites and also would induce adjacent host tissue to form additional mesodermal and ectodermal structures. Host and transplant structures supplemented each other in the formation of a whole new organism This capacity for integrated inductions has earned for the upper

lip the designation "organizer The further analysis was greatly en hanced by the application of 'heteroplastic transplantation i e ex change between embryos belonging to two different species making it possible to distinguish between self-differentiating donor tissue and induced host tissue

In the organizer action the following components can be distinguished

1 The transplanted upper lip invaginates. It possesses autonomous gastrulation tendencies

2 It self-differentiates into notochord and somites or other mesodermal tissue

3 The transplant induces adjacent host mesoderm to form mesodermal structures such as notochord, somites pronephros lateral plate etc Host and transplant structures supplement one another to form a com plete set of mesodermal axial and paraxial organs The share of the trans-plant in this secondary set is variable and depends partly on the initial size of the transplant.

4 The transplant induces overlying host ectoderm to form a neural tube which is frequently subdivided into brain with optic vesicles spinal cord etc

5 Occasionally a secondary gut is induced in host entoderm

In the following a few points will be discussed which are of importance in planning the experimental work Bautzmann (1926) has shown that the region of the early gastrula which possesses organizer capacity corre sponds to the chorda mesoderm area (see map Fig 4) Therefore it is not necessary to use strictly median parts of the upper lip The results of the experiment will differ when upper blastopore lips from different stages of gastrulation are used It will be remembered that the upper lip of the early gastrula is composed of material which invaginates first and will come to be head mesoderm The upper lip of late gastrulae is prospective trunk mesoderm Spemann (1931) has shown that the two differ in their inductive capacities the former has a tendency to induce head structures and is therefore called 'head organizer and the latter the trunk or ganizer tends to induce trunk structures It is therefore necessary to check and to protocol carefully the developmental stage of the donor Furthermore, the host level to which the transplantation is made influ ences the result Spemann (1931) has found that the head-organizer tend encies are strong enough to induce a head in any host level However the trunk organizer will induce a trunk only in the trunk level whereas it will induce head structures in the head level In the latter instance the host influence overrides the inherent tendency of the transplant. Since many of the inductions obtained by the student will be partial rather than com

plete embryos these findings may help to interpret the results Finally the axial orientation of the induced embryo will be found to vary considerably the axes of the host and of the induced embryo may be at any angle with each other Again Spemann (1931) has found that the transplant has its intrinsic polarity bnt that the more powerful gastrulation movements of the host will cause those of the transplant to deviate, so that the final orientation of the induced embryo will be either parallel to the bost axis or the resultant between the two tendencies

Altogether the most complete secondary embryos can be obtained when large median pieces of the upper lip of early gastrulae are implanted in the ventral lip opposite to the upper lip of the host (Fig 27 *b*) In this instance the directions of invagination of the host and transplant will be parallel and bost and donor structures will be in corresponding levels In this way the host will interfere little with the formation of the second ary embryo

EXPERIMENT 35

Material

gastrulae of *Ambystoma*, any species stages H10–H11
standard equipment (p 41)

Note—Use dishes with agar bottom for operations and for raising (p 11) Sterilize all instruments carefully

Procedure

Note—Since the color differences between donor and host are usually not very striking the transplant will soon be lost sight of unless it is vital stained Therefore vital staining of the donor embryos *in toto* in 0 1 per cent Nile blue sulphate is advisable. Remember that early gastrulae are very delicate when taken out of their membranes and that your success will depend on careful handling and working under sterile conditions

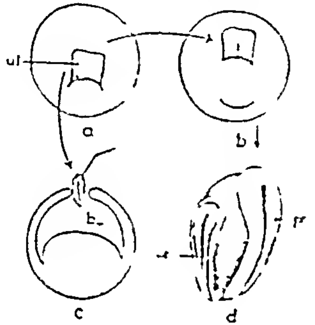
1 Select 8–12 healthy gastrulae In the stage of the sickle-shaped blastopore (preferably stage H10½ or stage H10¾)

2 *Remove all membranes*—Strip off the outer jelly membranes with two pairs of watchmaker forceps with not too sharp points. Wash the embryos several times in $\frac{1}{6}$ Holtfreter solution Transfer these to a dish containing sterile $\frac{1}{10}$ Holtfreter solution Remove the vitelline membrane (follow the technique described on p 39) Transfer the embryos to full strength Holtfreter solution Keep the dish tightly closed avoid any shaking of the dish

3 Prepare the operation dish fill it with full strength Holtfreter solu tion and prepare several glass bridges Make a smooth flat wide groove in the agar it should be larger than the embryo If the hole is too small

it is difficult to remove the embryo uninjured. Transfer embryos of the same stage into the dish. Use a sterile pipette which has not been used before for another purpose. Choose an embryo which has little or no injuries from the removal of the vitelline membrane. With the hair loop shove it into the groove (with very gentle movements). Place it upside down so that the upper lip faces you (Fig. 2, b).

4. *Prepare the hole in the host.*—With the tip of a very fine glass needle cut out a square area of the surface layer opposite to the blastopore ap-



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plant is opposite that of the host (Fig 27, *b*) Press the transplant gently into position, fit it in tightly, and enlarge the hole if necessary Push the glass bridge over the transplant (with 2 hair loops) and press it down so that the transplant fits well and is in the desired orientation. Discard the donor embryo Cover the operation dish at once and shove it gently aside, do not lift it up Healing takes ½–1 hour Control the progress of healing but disturb the embryo as little as possible. Readjust the glass bridge if necessary

7 After 45–60 minutes, cautiously remove the glass bridge Transfer the embryo in a clean sterile pipette to another dish with agar bottom filled with $\frac{1}{10}$ Holtfreter solution Dip the mouth of the pipette under the surface near the bottom before you release the embryo Place the embryo with both blastopores directed upward otherwise gastrulation will be impeded Cover the dish at once

8 Take a careful protocol, make sketches Indicate stage size, shape, orientation of transplant.

Note —Make 3–4 operations

9 During the following days disturb the embryo as little as possible handle it with utmost care After gastrulation is completed turn it right side up to allow neural folds to develop normally Watch for a secondary neural plate Make sketches

10 The induced embryo is usually at its best in stages during and after the closure of the neural folds (Fig 27, *d*) and in earliest tail bud stages. Study and draw these stages carefully The early tail bud stage is a critical stage, from then on mortality is high and only a few embryos will survive Fix in stage H20 or stage H21 If you wish to rear an embryo longer watch it every few hours for the first signs of disintegration or edema and fix at once Disintegration proceeds very rapidly once it has started

f) IMPLANTATION OF INDUCTORS IN THE BLASTOCOELE

(After Spemann and O Mangold)

Spemann and O Mangold found that a piece of the organizer when placed in the blastocoele of a blastula will be shifted into the region of the liver primordium by the gastrulation movements of the host and will induce a secondary embryo in this position (*Einsteck* method) In some instances it will be found in other locations Its final location cannot be determined exactly by the experimenter This disadvantage, however is outweighed by the advantages of this technique. It is a much simpler and faster experiment than the transplantation Furthermore it makes it possible to test the inductive capacity of structures which cannot be transplanted to the surface of the gastrula—for instance adult tissues

killed tissues or even foreign bodies or extracts or chemical substances which one can adsorb to agar and implant in this way The rapid progress in the organizer analysis and the discovery that medullary induction is mediated by a chemical substance were greatly enhanced by this technical advance

EXPERIMENT 36

Material

Ambystoma any species stages H10–H11 as donors stage H9 as hosts
standard equipment (p 41)
operation dish with agar bottom

Procedure

1–3 As in Experiment 35 Place one donor and one host in the operation dish. Operate in full strength Holtfreter solution.

4 *Prepare the host* —Place the host in a wide groove with the animal pole upward With the point of the glass needle make a slit in the roof of the blastula near the animal pole (Fig 27 *c*) Destroy as few cells as possible

5 *Cut out the transplant i.e a piece of the upper lip* (*Fig 27 a*) —Use the hair loop and glass needle. Spemann has frequently used the hair loop as a cutting instrument. It gives an oval transplant with sharp edges.

6 *Implantation* —Place the transplant near the slit in the host and work it through the slit with the tip of the glass needle (Fig 27 *c*) Push it deep down and try to bring the edges of the slit together Place a glass bridge over the wound and apply slight pressure When the edges of the wound fit well together it is advisable not to use a glass bridge at all

7 The host has a tendency to extrude the transplant, unless it is pushed into a deep position. Watch the operated embryos closely and reimplant if necessary Do not shake the operation dish

8 After healing is completed transfer the embryo to a dish with $\frac{1}{10}$ Holtfreter solution Take protocols

Make a number of operations The mortality is high Keep all dishes tightly closed Use sterile pipettes and hair loops for inspection

Sections 9 and 10 as in Experiment 35

EXPERIMENT 36a

Study the papers of Holtfreter (1934*a* and *b*) and try to obtain inductions with living or dead tissue of adult salamanders (brain, retina) or with pieces of medullary plate or ectoderm of *Ambystoma* which have been killed by heat or in alcohol (wash carefully before implanting) There is

a strong tendency for such implants to be extruded Healing has to be watched closely Do not try these experiments until you have had success with Experiment 36

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7 PARABIOSIS

(After R. K. Burns, Jr 1925)

This method consists of fusing together 2 whole embryos side by side or in other positions and allowing them to develop as conjoined twins. This can be accomplished readily by creating wound surfaces on the adjacent flanks and pressing them together until healing is completed Such twins have been reared through metamorphosis.

The method has been found useful for several purposes Harrison (1908 1924) in an experiment in which the fate of the neural crest was studied, healed together 2 frog larvae from which the dorsal halves of the spinal cord were removed to prevent their regeneration Detwiler (1926) in his analysis of the influence of peripheral fields on the development of the central nervous system used parabiotic twins to obtain a substantial skin loss without a proportionate muscle loss The most fruitful application of the method has been in the field of physiology of sex determination Conjoined twins share a common blood circulation and their sex hormones are distributed to both partners Since 50 per cent of all parabiotic twins are expected to be male female combinations valuable material can thus be obtained for the study of the interaction between genetic and hormonal sex determiners Burns and Witschi (reviews in 1934 1939) have applied this technique extensively for investigations of this problem Witschi has occasionally used an end to-end fusion of embryos (telobiosis)

Material

urodele larvae, stages H22–H28

anuran larvae stages PM16 or PM17 (embryos in later stages are motile and more difficult to handle)

standard equipment (p 41)

Procedure

1 With the glass rod with ball tip make a deep groove in the Permoplast wide enough to hold both embryos when pressed together and when in upside-down position. Smooth the edges Fill the dish with full strength Holtfreter solution

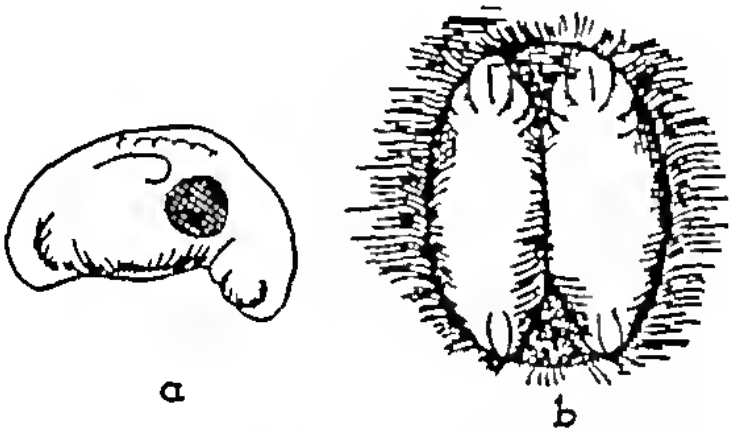

Fig. 28.—Parabiosis experiment (modified after Adams, 1941) *a* = embryo in operation stage cross-hatched = skin area to be removed from left twin embryo (see text) *b* = the two embryos in the operation groove.

2 Take a considerable number of embryos of identical stages out of all membranes

3 With the glass needle cut out a circular area of ectoderm of considerable extent on the left flank of one embryo and on the right flank of another embryo (Fig 28 *a*) Remove either the skin covering the gill swelling or the skin behind the limb and pronephros swelling

4 Place both embryos in the groove in upside-down position and with the wound surfaces adjacent to each other Partially imbed the embryos in Permoplast by building it up on the sides (Fig 28 *b*) Place a glass bridge on top of the embryos to hold them in position Otherwise they will move by ciliary motion which is rather strong in these stages Keep the embryos in the operation groove for 1 hour or longer

5 After 1–3 hours transfer the embryos to a glass dish or Lily cup in ⅓ Holtfreter solution

6 Observe the development of the twins their heart beat swimming

reactions etc. If time permits, raise the twins to stages in which the gonads are differentiated Dissect the gonads and compare them with normal gonads Sectioning is necessary for detailed studies Consult Witschi (1934 1939)

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8. EXTERNAL FACTORS IN DEVELOPMENT

a) THE PRODUCTION OF CYCLOPIA AND OTHER ABNORMALITIES BY TREATMENT WITH LITHIUM CHLORIDE

(After T S Hall)

Embryos will tolerate moderate changes in their environments but excessive changes in external factors such as temperature chemical substrate etc. will result in abnormal development Since external agents permit a quantitative approach they are a valuable tool in developmental mechanics

Of the large body of material which is available only two experiments were selected They concern the effects of changes in the chemical composition of the culture medium on the development of amphibian embryos. They illustrate certain general aspects of the role of external agents in development. Experiments on the temperature effect on development can be set up easily and require no special treatment Many other experiments concerning the role of light, of oxygen tension and other chemical properties of the milieu, of gravity etc can be planned by the student on the basis of literature studies

The peculiar effect of lithium chloride on early development was discovered by Herbst (1893) in his systematic study of the effects of certain

ions on sea urchin development. Lithium chloride if added to the sea water in a certain concentration and if applied before gastrulation results in exogastrulation that is the entoderm grows to disproportionately large size and instead of invaginating evaginates. This interesting phenomenon of entodermization in the sea urchin embryo has been thoroughly analyzed by Lindahl (1940) Child (1940) and others These studies give an insight into the physiological mechanism of lithium action and should therefore be consulted in connection with the amphibian experiments to be discussed here

The lithium effect on amphibians has been studied by Adelmann (1934 1936) Lehmann (1937, 1938) T S Hall (1942) and others If early gastrulae are kept in a high concentration of lithium chloride for several hours, exogastrulation results. The invagination of mesoderm and entoderm is inhibited (see p 122) If a lower concentration is applied for 6–24 hours then a variety of abnormalities results Most common among them are complex deficiencies of the head In the least affected cases the head is disproportionately small (microcephaly) Its bilateral structures (nose eyes forebrain hemispheres suckers or balancers) may be found in all degrees of approximation Extreme cases of this type may show an unpaired single nose (monorhyny) a single median eye (cyclopia) or even a complete suppression of any or all of these structures These monsters are strikingly similar to monsters found occasionally in human and mammalian fetuses Microscopic studies show that internal head structures—for instance the mandibular arch and the pharynx—are also affected Obviously the median part of the head is more severely affected than the lateral parts and the head as a whole is more severely affected than posterior regions. Different structures show a differential susceptibility to the lithium action Adelmann Lehmann and Hall each have demonstrated that the primary effect is on the median strip of the mesoderm (organizer) and that the effects on the ectodermal structures are secondary effects caused by faulty inductions. Lehmann applied the lithium treatment at different stages of gastrulation and showed that different regions of the organizer are differentially susceptible at different times For instance in early gastrulation the posterior head and anterior trunk level are the most highly affected regions Microcephaly and cyclopia were more frequent after treatment of the middle gastrula Variations of the concentration and of the time of exposure will likewise modify the effects (Hall 1942)

Obviously the same agent can produce a number of different end effects depending on its concentration on the duration of its application on the phase of development etc (see Stockard 1921) The specificity

of the end effects appears to be due to disturbances of the delicately balanced intrinsic developmental pattern rather than to the chemical properties of the lithium chloride This point is emphasized further by the fact that the same abnormalities (microcephaly, cyclopia etc) can be produced by a variety of other agents as well (Bellamy, 1919, 1922), for instance, by hypertonic sodium chloride solution, by magnesium chloride, etc. or by high temperature (Hoadley 1938)

However it would be misleading to make a sharp distinction between "nonspecific ' external agents and a specific ' intrinsic developmental pattern. If an external agent affects a fundamental physiological activity of all cells then its effects are widespread, and the end effect depends largely on the *differential susceptibility* of the organ primordia—the agent will give the impression of a "nonspecific ' entity If by virtue of its chemical properties, it affects selectively a local process, then it will appear as a 'specific agent. In the last analysis differentiation is brought about by a complex interplay of intrinsic and extrinsic conditions, and the term "specific is dispensable

EXPERIMENT 38

Material

beginning gastrulae of *R. pipiens* or *R sylvatica* or *R. damilans* or *R catesbeiana* or *A maculatum* or *A tigrinum*
glass jars or Lily cups with tightly fitting lids
pipettes
lithium chloride c.p , 1 9 gm. in 1,000 ml. distilled water
lithium chloride, c.p , 1 9 gm in 250 ml. distilled water

Note —Concentrations and time of exposure which are necessary to produce microcephaly vary slightly with the species used The following data apply to *R pipiens* Considerable variations will be found, even if the material is treated uniformly If you find exogastrulae, consult section *b* (p 122) If possible keep the temperature constant (optimal temperatures between 18° and 22 C)

Procedure[1]

1 Remove all membranes except the vitelline membrane, from 100–200 eggs

2 Place 30–50 embryos (shortly after appearance of the blastopore) into each of the following solutions

a) 1 9 gm LiCl c.p in 1 000 ml. distilled water
b) 1 9 gm LiCl c p in 250 ml distilled water

[1] I am greatly indebted to Dr T S Hall for a personal communication of the technical details of this experiment previous to their publication.

c) $\frac{1}{10}$ Holtfreter solution (control)

Do not crowd the eggs Cover the dishes

3 After 24 hours transfer the embryos from solution *a* to $\frac{1}{5}$ Holtfreter solution After 2–7 hours transfer part of the embryos from solution *b* to $\frac{1}{5}$ Holtfreter solution leave the rest of the embryos in solution *b* for 24 hours Take careful protocols of the time of exposure etc. Label all dishes carefully

4. Observe the gastrulation and following stages Note and sketch the first deviations from normal development

5 After the swimming stage is reached (eyes pigmented) fix all embryos in 10 per cent formaldehyde Dissect the most interesting cases with glass needle and watchmaker forceps Compare with normal embryos Notice the great variations in the effect even within the same group of embryos Arrange all cases in a graded series Record your results in a tabulated form (consult Hall 1942)

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b) EXOGASTRULATION AND SPINA BIFIDA PRODUCED BY TREATMENT WITH HYPERTONIC SALT SOLUTION

(After Holtfreter)

If amphibian embryos are exposed to a hypertonic salt solution before the onset of gastrulation the entoderm and mesoderm fail to invaginate. Instead they evaginate and a constriction appears between the ectoderm and the partly or fully evaginated inner germ layers (Fig 29 *a*)

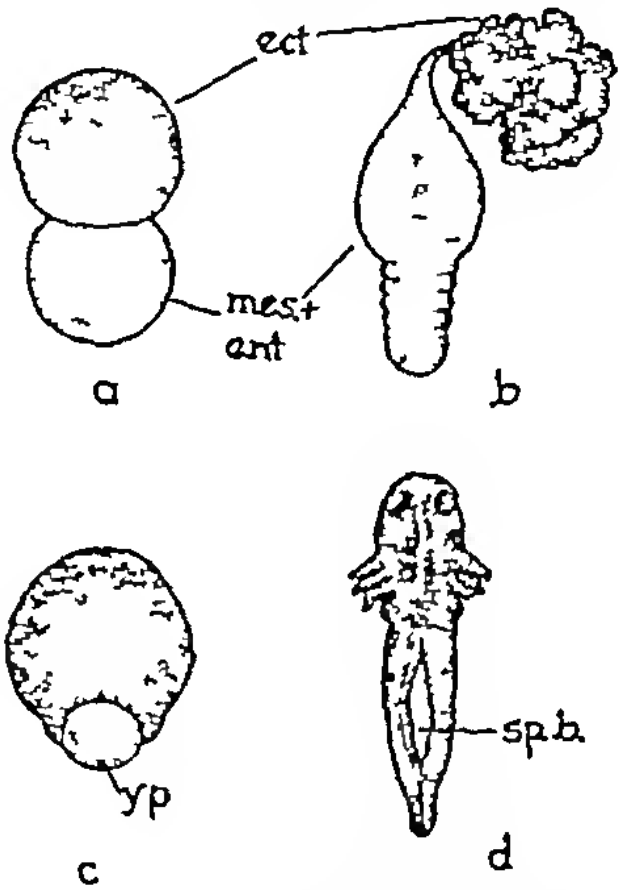

FIG. 29.—Total and partial exogastrulation (from Holtfreter 1933*c*) *a b* = total exogastrulation *a* = in gastrula stage *b* = in tail-bud stage *ect* = ectoderm *mes+ent* = evaginated mesoderm + entoderm *c d* = partial exogastrulation *yp* = yolk plug which fails to invaginate *sp.b* = spina bifida (open spinal cord and vertebrae)

Exogastrulation is one of the most common disturbances of early amphibian development and can be produced in many ways such as by treatment with lithium chloride or with magnesium chloride etc. An exhaustive analysis of exogastrulation in amphibians was given by Holtfreter (1933*b*) who applied a dilute Ringer solution (0 35 per cent this is the so-called "Holtfreter solution") His paper contains a discussion and bibliography of earlier work. His experiments are reviewed extensively in the Appendix of Huxley and De Beer (1934)

Holtfreter obtained a graded series of exogastrulae Total exogastrulation (Fig 29 *a b*) in which the ectoderm is separated almost completely from the entoderm and mesoderm is rare. In partial exogastrulation the anterior head organizer and head entoderm fail to invaginate As a result parts of the head are not differentiated In the least affected cases (Fig 29 *c*) the large yolk plug merely fails to be absorbed The fusion of the medullary folds which differentiate at its circumference is blocked In later stages this deficiency appears as a slit in the spinal cord (it is known as ' spina bifida [Fig 29 *d*]) Holtfreter followed the evagination movements by means of vital stain marks and showed that the morphogenetic movements described by Vogt (pp 51 ff) proceed in a typical fashion though in reversed direction This indicates a very early determination of the gastrulation movements. Holtfreter succeeded in raising complete exogastrulae to advanced stages In all instances the entomesodermal part proceeded surprisingly far in its differentiation in spite of the absence of the ectodermal covering and of all nervous structures and in spite of the complete inversion of all structures (entoderm ontside mesoderm inside) The ectodermal part however failed to undergo any differentiation (Fig 29 *b*) This strongly supports the contention that inductive stimuli from the mesoderm are necessary for neural differentiation

It is very difficult to raise exogastrulae beyond the gastrulation stages therefore the following observations will be concerned mostly with a study of the atypical gastrulation itself

Experiment 39

Material

Ambystoma any species stage H7 or stage H8 (The experiment is unsuccessful if early gastrulae are used)

glass dishes or Lily cups with lids full strength Holtfreter solution

pipettes $\frac{1}{3}$ Holtfreter solution

Procedure

1 Remove all membranes from 20 30 eggs Holtfreter removed the vitelline membrane also However this is difficult to do without injury to the embryo It is therefore recommended that it be left intact

2 Place two-thirds of the embryos in full strength Holtfreter solution and the others in $\frac{1}{3}$ Holtfreter solution as controls Leave them in this solution until the controls are in the neurula stage Observe the process of exogastrulation A considerable variation of the effect will be noticed Isolate and draw the most interesting cases Tabulate your results Rare embryos which show slight degrees of exogastrulation (Fig 29 *c*) to swimming stages and observe spina bifida (Fig 29 *d*)

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9 THE DEVELOPMENT OF BEHAVIOR PATTERNS

a) THE ORIGIN OF EARLY REFLEXES
(After Coghill)

Behavior patterns like organs have an ontogenetic development. The development of behavior is studied best in the simple reflexes of lower vertebrate embryos. The classical work of G E Coghill on the early reflexes of salamander larvae has led him to conclusions of great importance for biology and psychology Some of his observations on *Amblystoma* larvae can easily be repeated and are therefore introduced here. His series of lectures *Anatomy and the problem of behaviour* (1929) should be consulted in connection with this exercise.

The swimming reflex is the first integrated activity of an amphibian larva. Its origin was studied by Coghill with the interest focused on the problem Is this reflex the result of 'learning or is it entirely the result of the maturation of the nervous system with no contribution from 'experience" and external stimuli?

Swimming is preceded by spontaneous wriggling motions within the membranes long before hatching Coghill studied in detail these earliest reflexes and classified them in a number of behavior stages (p 125) This series shows clearly the progression in the complexity of the behavior pattern. A slight bending of the head is the first perceptible movement. It is followed by a more intense bend, or coil, extending from the head tailward. The addition of a second coil in the opposite direction before the first has reached the tail results in an S-like wriggle the swimming emerges from this double flexure as a series of continuous and more powerful S-reactions Coghill succeeded in accounting for each step in this behavior development in terms of a stepwise increase in the complexity of the nervous system. With each stage a new type of neurons, or connections, is added. The following experiments and observations obtain their full significance only in the light of these neurological data. The student

should study the diagrams of the organization of the nervous system in different stages of behavior (in Coghill 1929)

The general conclusions at which Coghill arrived have influenced and modified considerably our concepts concerning the origin of behavior and of reflexes. In contradiction to the views held by many psychologists and biologists he demonstrated at least for his object that complex behavior patterns do not originate by assembling separate simple reflexes and integrating them secondarily but that the reverse is true all activities are integrated first and each step emerges from the preceding one as an integrated unit Local reflexes are secondary emancipations from an integrated total pattern The origin of the swimming reflex illustrates this principle. The origin of independent limb movements is another example To quote Coghill

> The first movement of the fore limb is adduction and abduction. When this movement of the limb is first performed it occurs only with trunk movement. When the trunk acts vigorously as in swimming the fore limbs are drawn close against the body
>
> A day or two ordinarily elapses between the time when the arm begins to move with the action of the trunk before it acquires the ability to respond to a local stimulus without the perceptible action of the trunk. It is obvious, therefore, that the first limb movement is an integral part of the total reaction of the animal, and that it is only later that the limb acquires an individuality of its own in behaviour [1929 pp. 18 f].

The same holds for jaw movements in feeding gill movements etc Altogether complex activities such as swimming or feeding are not the result of experience or learning but the result of an orderly sequence of developmental steps of the neural mechanisms

BEHAVIOR STAGES

These stages were worked out by Coghill The following definitions are taken verbatim from DuShane and Hutchinson (1941 pp 250-51) with a few additions

NR. Premotile stage —No response to repeated touch and deep pressure on the myotomes

NM The nonmotile or myotomic response —This occurs in the absence of and earlier than the ' early flexure It is characteristically a slow contraction toward the side stimulated followed by a slow relaxation It begins with a bending of the head This is regarded as a direct nonnervous response of the myotomes

EF The early flexure response —This is a rather rapid reflex response of the animal to gentle touch The bending of the body beginning at the head is always away from the side stimulated (contralateral) The reac

tion is brief in duration, the relaxation being abrupt. With further development of the myotomes and the nervous system, the contracted phase tends to be held for a longer period of time and the tail is brought progressively nearer the head There is no sharp natural distinction between the more advanced flexures and the next stage.

Coil —The coil reaction is aptly named It is the culmination of the early flexure and is attained when the tail touches or passes the head at the height of the response It is again away from the stimulated side (i.e. contralateral) Some embryos show the coil reaction in typical fashion followed by a coil in the opposite direction without additional external stimulation.

S-reaction —This is a reaction superimposed upon the coil reaction It results when a wave of contraction passes down the stimulated side before the original contralateral contraction has relaxed The embryo is transitorily in the form of the letter S Occasionally in response to a single touch the reaction may be repeated several times successively but it does not yet result in locomotion

ES The early-swimming response —Repeated S-reactions become so organized and strengthened that the embryo makes some forward progress. Embryos which show any progression not more than approximately 3 body lengths fall into this category

SS Strong swimmers —Embryos which swim for more than 3 body lengths to less than 10 body lengths

LS Late swimmers —Embryos which swim 10 or more lengths

Experiment 40 Observations on the Earliest Reflexes of *Amblystoma* Larvae

Material

embryos of any species of *Ambystoma* in all stages from H31 to H46
dishes
a hair loop or a fine hair or bristle mounted in a glass handle

Procedure

Choose embryos of different stages. Place one after another in a dish. Make all observations under the binocular microscope With the hair loop stroke the embryo gently along the row of the right myotomes Observe the reaction as carefully as possible Make a record of the reaction in terms of the behavior stages listed above and of the Harrison stages Repeat these observations on a considerable number of embryos and find representative specimens for each Coghill stage Try to correlate the behavior stages with the Harrison stages You will observe that there is a considerable variation of behavior reactions in embryos of the same Har

rison stage. For instance embryos in stage H35 may exhibit any of the following reflex responses *NM EF* Coil S Such variations may be found even in material from the same lot of eggs. DuShane and Hutchinson (1941) have devoted a special investigation to this variability They have given precise data for the range of variation of behavior stages in terms of Harrison stages for two different temperatures using *A maculatum* The results of the class should be compiled and compared with Table 1 and Figure 1 of the paper quoted above

b) THE DEVELOPMENT OF REFLEX ACTIVITY IN NARCOTIZED EMBRYOS

(After Matthews and Detwiler 1926)

It is possible to give experimental evidence for the contention that experience plays no role in the formation of the swimming reflex of salamanders In connection with another study Harrison (1904) placed frog embryos of early tail bud stages—i e previous to the first movements—in chloretone and kept them in a narcotized condition for as long as 7 days They were returned to normal water at a stage when the controls were swimming larvae After a short period of recovery they began to swim normally Obviously the nervous system and also the musculature had developed normally in complete absence of functional activity Later experiments along similar lines have shown that we are dealing here with a general principle of development which holds for organs and structures as well as for the ontogeny of behavior Most structures such as the eye or the kidney are differentiated first and begin to function later

The narcotization experiments of Harrison were repeated on *Ambystoma* embryos by Matthews and Detwiler (1926) The following experiment is based on this paper which should be consulted for details

EXPERIMENT 41

Material

Ambystoma any species stage H28 or stage H29
Petri dishes or section dishes with tightly fitting lids
chloretone 1 3 000 or MS 222 1 6 000 in $\frac{1}{4}$ Holtfreter solution
hair loop

Note—The mortality in narcotics is high Different batches of eggs may require different concentrations The appropriate concentration should be tested in advance

Procedure

1 Select 15 healthy embryos in a stage preceding the onset of muscular movements (H28 or H29) Remove all membranes Place 10 specimens

in a dish with one of the narcotics mentioned above, keep 5 embryos in $\frac{1}{4}$ Holtfreter solution as controls Cover both dishes

2 During the following week change the narcotic daily keep the dishes tightly covered to prevent evaporation, remove all dead animals. By gently stroking the narcotized animals with the hair loop, check each day to see if they are completely immobilized If not, transfer them to a stronger concentration

3 When the embryos have reached stages H38–H40 and the controls are swimming larvae, transfer the narcotized embryos to $\frac{1}{4}$ Holtfreter solution Watch their recovery Stimulate with the hair loop Take notes.

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PART III

EXPERIMENTS ON THE CHICK EMBRYO

A MATERIAL AND TECHNICAL PROCEDURES

1 LIVING MATERIAL INCUBATION

The chick embryo ranks second to the amphibian embryo as a material for the experimental analysis of embryonic development. During the past decades the classical methods of extirpation transplantation explantation and vital staining have been applied successfully to the chick. In addition the method of chorio-allantoic grafting has given valuable information concerning the potencies of early primordia. The availability of eggs at almost any time of the year and the short duration of early development are great advantages in experimental work.

Data on different breeds on the principles and practice of incubation on factors influencing hatchability etc., are easily accessible and will not be presented here. Full information may be found in Jull (1938) and Lippincott and Card (1939). The following remarks will be limited to a few essential details.

The most important prerequisite for successful operations is a supply of first rate strictly fresh eggs with a high percentage of fertility and a low percentage of abnormal development. The quality of the eggs should be tested rigorously before operations are started on a large scale. There is apparently no difference in quality between the different breeds.

Storage.—Eggs should not be stored longer than 6 days; they deteriorate when they are older. They should be stored in a cool place. The optimal temperature for storage is 55° F.

Incubators.—Two types are on the market: models without forced air draft in which the warm air diffuses downward onto the egg; and models with forced air circulation. Smaller units are usually of the first type. They are entirely satisfactory for laboratory use but require more attention than the latter. Incubators are best installed in a room with even temperature and with good ventilation. They should not be exposed to direct sunlight. It is advisable to follow closely the directions of the manufacturer concerning temperature, humidity, etc.

Temperature.—In the still-air type the temperature varies considerably in different levels of the incubator space. The temperatures 2–3 inches above the eggs (readings on hanging thermometers) are 1°–2° higher than temperatures on top of the eggs. The optimal temperature for incubators without forced air draft is 100° F. throughout the incuba-

tion period (readings on thermometers which are placed on top of the eggs)

Humidity—Humidity is a very essential factor in successful incubation Refilling of the water pans or of other devices for evaporation should be carefully attended to In general a relative humidity of 60 per cent was found to be optimal The humidity in the incubator is of course, closely related to the humidity in the room and to the ventilation within the incubator and in the room

Turning—Eggs should be turned twice a day

Fertility, hatchability, mortality—The natural breeding season is February to June, and the best material can be obtained during this season but acceptable material can be obtained at almost any time except during hot summer months Very cold weather reduces the percentage of fertility Fertility and hatchability are not correlated with each other A hatchability of 80 per cent, which implies an even higher percentage of fertility, is considered satisfactory Two peaks of mortality occur during incubation, one around the third or fourth day and another around the eighteenth to twentieth day (see Landauer, 1941)

Testing by candling—Candles can be obtained from farm supply houses, or they can be easily prepared in the following way Make a circular hole approximately 2 inches in diameter in the bottom of a tin can. Mount a 100-watt bulb on a wooden base and invert the tin can over it Place the egg over the hole. Candle in a darkened room. The yolk sac circulation becomes visible at 2½–3 days of incubation as a network of blood vessels radiating from an indistinct dark spot which is the embryo In the following days the rocking movements of the embryo can be recognized in candling and the expanding vitelline circulation as well as the beginning chorio-allantoic circulation can be seen. From the third to about the seventh day dead embryos can be recognized by the "blood ring"—blood settles at the periphery of the area vasculosa. From the seventh day on the chorio-allantoic circulation can be seen in live embryos as an irregular network closely applied to the shell. From the thirteenth day on living embryos appear increasingly dark and the line of demarcation against the air chamber is very sharp and distinct In embryos which die during these days this line is indistinct and hazy

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2. LIMB BUD STAGES

No satisfactory stage series of the chick exists that of Keibel and Abraham (1900) is not very useful Many textbooks particularly that of Lillie (1919) contain illustrations of different stages In most of the following experiments the limb buds of embryos of 48–72 hours of incubation are used for transplantation etc. To facilitate the quick identification of such embryos we distinguish the following stages which are based on somite numbers and on the shape of the wing buds (Hamburger 1939)

Stage 1 —A slight thickening of the somatopleure appears in the wing level (adjacent to somites 14–16) 24–27 somites Incubation period 48–55 hours

Stage 2 —The Wolffian ridge a thickening along the lateral border of the somites is visible in the wing level 27–28 somites Incubation period 51–56 hours

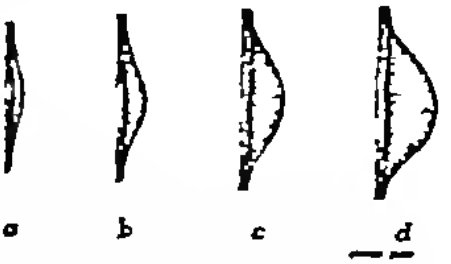

FIG 30 —Stages of wing buds of chick embryos (from Hamburger 1939) *a* = stage 3 *b* = stage 4 *c* = stage 5 *d* = stage 6 (see text)

Stage 3 (*Fig 30 a*) —Wing and leg primordia are marked off as slight swellings. 29–32 somites Incubation period 52–64 hours

Stage 4 (*Fig 30 b*) —Wings are small buds length width = 5 1 30–36 somites Incubation period 65–69 hours

Stage 5 (*Fig 30 c*) —Wings are median buds length width = 4 1 36 38 somites Incubation period 68–72 hours

Stage 6 (*Fig 30 d*) —Wings are large buds length width = 2 5 1 39–42 somites Incubation period 70–72 hours

Note —Stages 1 and 2 are difficult to handle as donors stage 6 is too old for donors but may be used as hosts

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3 STANDARD EQUIPMENT FOR OPERATIONS ON CHICK EMBRYOS

Material for joint use of the class

Incubator with turning trays and with hatching trays (wire trays) It is advisable to use two incubators one with wire trays in which the eggs on their nests can be placed temporarily during the operation and one with turning trays in which the operated eggs are

placed for further incubation The latter incubator should be opened as little as possible

1 autoclave

1 or more heating plates for warming the eggs during the operation

1 or more mounted candles (p 132)

several containers with melted paraffin and a small brush

cotton 'nests (Place a padding of cotton on a watch glass. Mold the cotton into a groove in which the egg will fit. Eggs are placed on these "nests during the operation)

agar plates stained with Nile blue sulphate and neutral red for vital staining (for preparation of the plates see p 56)

*several large 5 liter flasks with NaCl 0 9 per cent'

*paper towels

several buckets for discards

Each student needs

*5 watch glasses 3 with lids

1 small scalpel

1 hack saw blade

2 pairs of watchmaker forceps

1 iris knife or fine lancet (sharpened steel needle, Fig 1, *f*)

1 pair of fine scissors

1 pair of large scissors

6 square cover glasses, 12–15 mm

1 micropipette (p 4)

*1 pipette with very wide mouth

*several medicine droppers

1 water glass or jar with cotton on the bottom and half filled with 70 per cent alcohol for sterilization of fine steel instruments

4 THE LUNDVALL TECHNIQUE OF CARTILAGE STAINING *in toto*

(After Lundvall, Anat. Anz. 25 1904 27 1906)

Usually it is not feasible in courses of experimental embryology to section the transplants Therefore it is suggested that limb primordia be used for chorio-allantoic, coelomic and flank grafts Cartilaginous limb skeletons of transplants, 9–13 days old can easily be stained *in toto* within less than a week using the simple method described below For older, ossified skeletons the alizarin red method is recommended It is suggested that normal (host) limbs be stained together with the transplants for comparison

All items marked with an asterisk ( ) must be autoclaved.

1 Fix in Bouin's fluid

picric acid, sat. aq. sol.	75 parts
40 per cent formaldehyde	25
glacial acetic acid	5

or in formaldehyde 1 10 for 1–2 days.

2 Wash in 70 per cent alcohol After Bouin fixation add a few drops of lithium carbonate (saturated solution in 70 per cent alcohol) Change the fluid until the yellow color has completely disappeared

3 Remove skin with feathers adhering viscera and fat masses Use a watchmaker forceps

4 Stain in methylene blue (0 25 gm per 100 cc. of 70 per cent alcohol with 3 per cent of HCl by volume) or in toluidin blue (same solution) for 2–3 days

5 Destain in 70 per cent alcohol for at least 48 hours (change several times) in 95 per cent alcohol for 3–4 hours (change) and in absolute alcohol for 12 hours (change) In absolute alcohol the soft tissues should be completely destained, but the blue cartilage can be seen faintly through the other tissues

6 Clear harden and store in 3 parts of oil of wintergreen *plus* 1 part benzyl benzoate.

B EXPERIMENTS

1 THE PROSPECTIVE SIGNIFICANCE OF EMBRYONIC AREAS (VITAL-STAINING EXPERIMENTS)

a) PROSPECTIVE ORGAN FORMING AREAS AND MORPHOGENETIC MOVEMENTS IN THE CHICK

Before starting the following experiments the student should acquaint himself thoroughly with the normal development of the chick embryo, from the primitive streak stage to the early somite stage (between 12 and 36 hours of incubation)

The experiments suggested below do not deal with the two earliest phases of gastrulation, namely, the formation of the entoderm and the formation of the primitive streak itself Both phases, though of great interest, are not favorable for classroom experiments. Our starting-point is the stage of the definitive primitive streak (about 16–18 hours of incubation) The primitive streak itself is a thickening of the upper layer, extending anteriorly from near the posterior margin of the oval area pellucida for about two-thirds of its length (Fig 33 *a*) The anterior end of the primitive streak is a knoblike thickening, called "Hensen's node." The streak is the region of mesoderm invagination It is formed of densely packed embryonic cells which continue laterally as organized epithelia both in the ectodermal and in the mesodermal layer The underlying entoderm applies itself closely to the streak, particularly in the node level yet it remains a separate unit The streak and the adjacent regions contain the material for all structures of the embryo proper with the exception of the forebrain portion of the head which originates from material lying in front of Hensen's node The peripheral regions of the area pellucida and the entire area opaca form extra-embryonic structures Shortly after the definitive streak stage is reached the differentiation of the embryo begins with the formation of the head process, which is an anterior extension in front of Hensen's node (Fig 32, *h p*) Sections show that the head process is the first visibly differentiated structure it is composed of the anterior end of the notochord and the overlying medullary plate From then on organ formation proceeds rapidly in anterior posterior direction Hensen's node recedes whereby the primitive streak shortens and at the same time the differentiated organized anterior part of the embryo

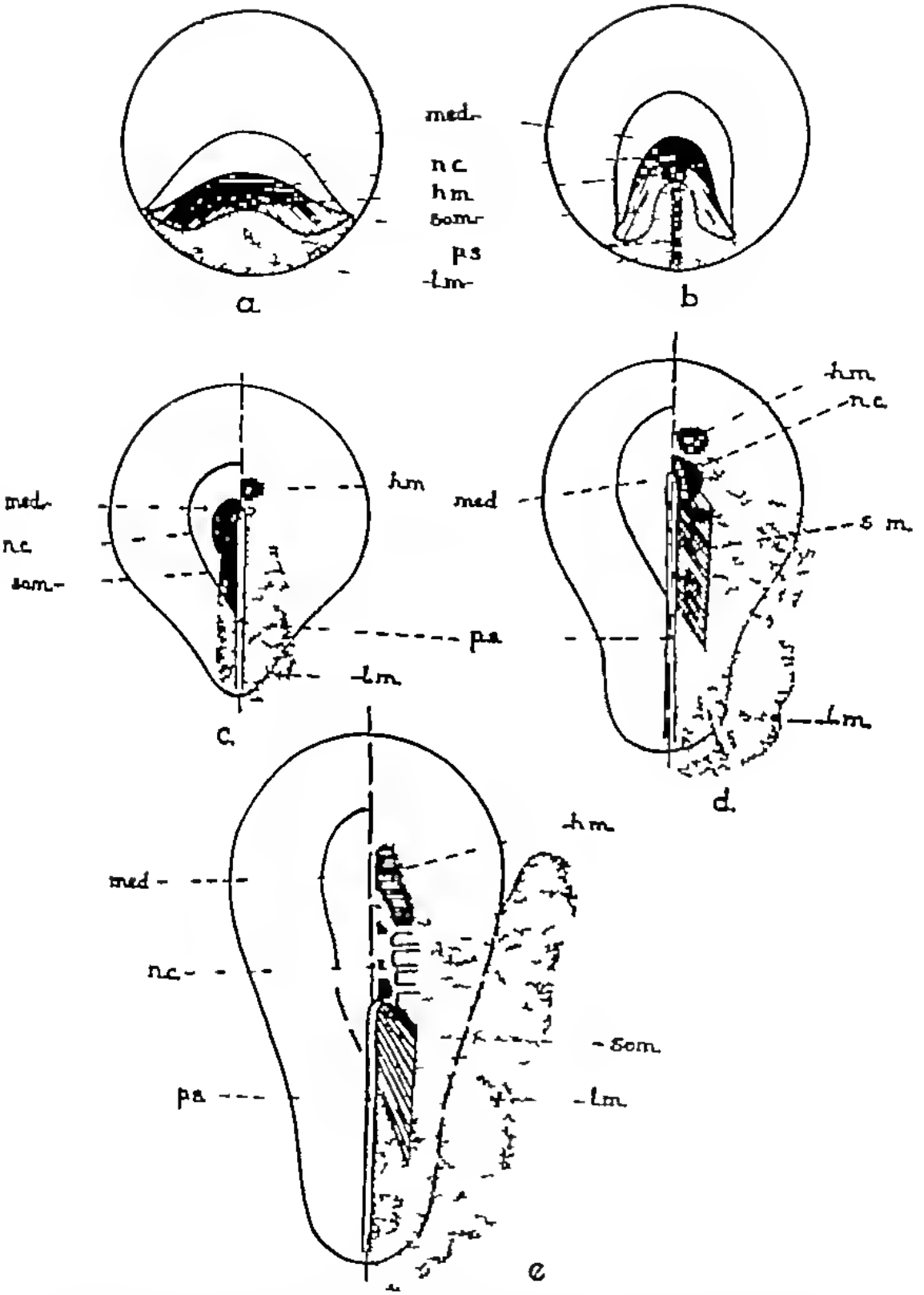

FIG. 31.—Maps of prospective organ forming areas in the chick embryo (from Pasteels 1937). *a* = early blastoderm. *b*, *c* = stages of the formation of the primitive streak. *d* = definitive primitive streak. *e* = head process stage. In *c*, *d*, *e* the left side shows the map of the surface layer and the right side shows the map of the segmented mesoderm. *h.m.* = head mesoderm. *l.m.* = lateral mesoderm. *med.* = medullary plate material. *c.* = notochord. *p.s.* = primitive streak. *som.* = somites.

lengthens (Fig 33, *c–e*) The anterior end of the neural tube closes, while the neural plate in more posterior levels begins to form One somite after another is laid down the notochord lengthens Hensen's node always marks the boundary between the anterior organized and the posterior unorganized part of the embryo

The localization of the prospective organ forming areas in the primitive streak stage has been mapped out by Wetzel (1929) and Pasteels (1937), using the vital-staining technique I have adopted as the basis for the following experiments the map of Pasteels, whose criticisms of Wetzel's map I consider as justified. The map of the definitive primitive streak stage (Fig 31 *c d*) can best be understood if one goes back to earlier stages The pattern of the different areas in a stage preceding the formation of the primitive streak is shown in Figure 31 *a* This stage corresponds to an amphibian blastula (except for the fact that, in the chick, entoderm formation is already completed the similarity of the maps for both forms is striking cf Fig 4) The morphogenetic movements during the subsequent phase of primitive-streak formation also have many features in common with those of the amphibian gastrulation, we find again the three basic movements convergence elongation and invagination All areas swing toward the midline (convergence), they elongate considerably and *mesoderm invaginates through the primitive streak* (the left half of Fig 31 *c–e*, is a surface view and the right half shows the mesoderm after removal of the ectoderm) The lateral and ventral mesoderm (*l.m*) is the first material to invaginate around the growing primitive streak (*p.s* in Fig 31 *b*) In the phase between stages *c* and *d* the prospective notochord material (*n.c*) invaginates around Hensen's node the prospective somite material (*som*) invaginates around the anterior *half of the* primitive streak and the lateral mesoderm continues to invaginate around the posterior half of the primitive streak. The prospective medullary material (*med*) converges toward the median line in conjunction with the adjacent mesoderm It arrives near the median line when stage *d* is reached However it is likely that left and right halves of the medullary plate do not fuse in the midline that is no concrescence takes place. The median strip of the medullary plate (future floor of the neural tube) is probably formed by material which, in the stage under discussion is located in Hensen's node above the notochord and which in subsequent stages moves backward and elongates enormously Both Wetzel and Pasteels found that when Hensen's node was vital stained deeply so as to stain both its ectodermal and its mesodermal component then the entire notochord as well as the entire length of the floor of the neural tube were

stained in the embryo This implies that Hensen s node is the actual material for the notochord and for the floor of the neural tube and that from stage *d* on Hensen s node moves backward and leaves in its wake the notochord and the floor of the neural tube. It diminishes in size while it recedes The regression of Hensen s node is part of a very conspicuous elongation in posterior direction of the posterior area pellucida, whereby the latter changes from a circular or oval to a pear shaped elongate form These movements are indicated by the arrows in Figure 32 The processes of convergence and of invagination are probably completed in stage *d* before the extensive elongation begins and the latter would then be a separate final phase of the morphogenetic gastrulation movements A peculiar feature of this spreading is that the median part (Hensen s node material) moves and elongates more rapidly than the adjacent lateral materials As a result the somites and the overlying medullary material which are at first staggered at the anterior part of the primitive streak unfold and spread in a fanlike fashion (Fig 31 *e*)

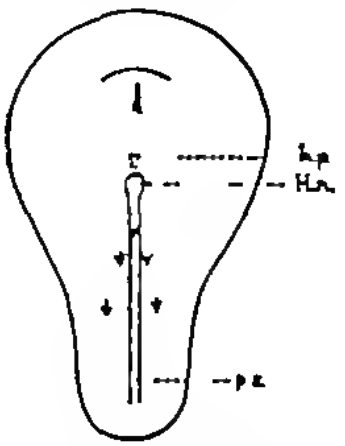

FIG 32 —Head process stage The arrows indicate the movements of Hensen s node and adjacent material. *H.n.* = Hensen s node *k.p* = head process *p.s* = primitive streak (after Pasteels, 1937)

According to this account, a vital stain mark placed on Hensen s node will result in a stain of the entire length of the notochord and of the floor of the neural tube. A mark placed on the anterior primitive streak (Fig 31, *d*) will stain somites lateral walls and roof of the neural tube The material for the anterior part of the head in primitive-streak stages is located in front of Hensen s node In order to stain the eye one must place a mark on the prenodal area slightly lateral to the median line It should be clearly understood that vital staining experiments concern only the prospective significance of organ forming areas The inherent potencies of the different regions are much greater as is shown by chorio-allantoic grafts and explantation experiments (see p 143)

The observations of Jacobson (938) on sectioned material are in part, at variance with this account. Jacobson points out a possible source of errors in vital staining experiments on the chick In the latter the vital dye is taken up by the yolk and fat globules and not by pigment granules as in the amphibians He found that in migrating materials (except notochord) lose their lipid content. As a result the dye may diffuse into other cells and thus give an erroneous picture of the actual cell movements However this source of error does not affect materially the interpretation of the three experiments suggested below

b) GENERAL PROCEDURE OF VITAL STAINING (EXPERIMENTS 42–44)

(After Wetzel and Pasteels)

Wetzel was the first to apply Vogt's technique of vital staining to the chick embryo The results are not so satisfactory as in amphibians. In the chick the stain is taken up by yolk and fat globules, which break down in cell metabolism As a result the marks fade out more rapidly than in amphibians, in which the pigment granules are the chief carriers of the stain It is therefore necessary to apply a deep stain. On the other hand, overstaining must be avoided, because it may result in a disintegration of the stained cells and in death of the embryo Vital stained embryos should be inspected not later than 24 hours after the experiment.

Material for Experiments 42–44

6–8 eggs per student
standard equipment (p 133)

Procedure

1 Incubate the eggs for 16–24 hours depending on the temperature and moisture of the incubator It is advisable to make preliminary checks of the rate of development under the particular conditions of the incubator used

2 Several hours before the laboratory period begins, autoclave the materials marked with an asterisk (p 134)

3 *Other preparations for the whole class* —Begin the heating of the warming plate and of the paraffin 1 hour before operations.

4 *Each student should make the following preparations* —Place forceps, scalpel, hack saw blade and scissors in 70 per cent alcohol 1 hour before operations start Prepare 2–3 nests by placing a padding of cotton on a watch glass and mold it into a depression into which the egg will fit. Fill 2 watch glasses with sterile saline and cover them Keep a third watch glass dry and uncovered Before the operations start, cover the table with sterile towels Wipe off all alcohol from the steel instruments and place them in a fold of a sterile towel, to the right of the binocular microscope. Moisten a small strip of the red and of the blue agar plate with saline solution After 1–2 minutes scrape off small strips of the swollen agar Place them in one of the watch glasses in saline solution and cut them into small square pieces.

5 Saw the window in the shell Candling is not necessary In these early stages the blastoderm will always float on the top of the yolk Place the egg on the cotton nest with the blunt end to your left, shake it gently to loosen the blastoderm In case it should stick to the membrane Saw

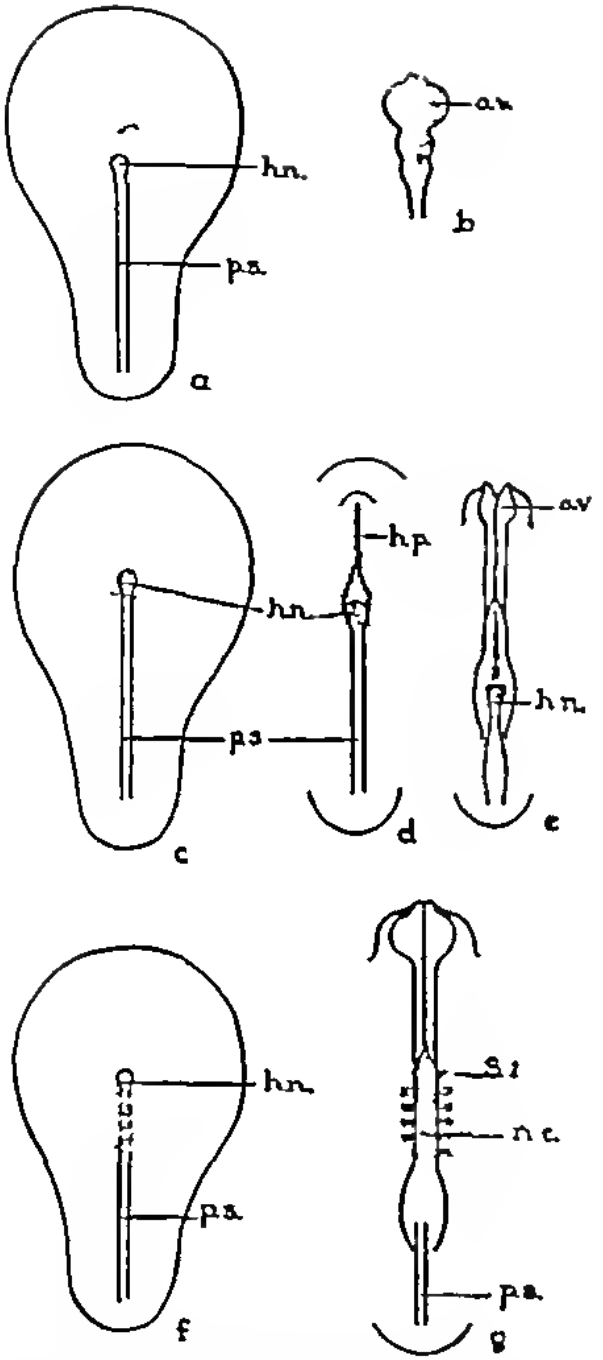

FIG. 33.—Vital-staining experiments in the primitive-streak stage (after Wetzel, 1929). *a*, *b* = vital staining of a region in front of Hensen's node (prospective right forebrain and right eye). *a* = immediately after staining. *b* = the same embryo 24 hours later. *c–e* = vital staining of Hensen's node. *c* = immediately after staining. *d* = 3 hours later. *e* = 24 hours later. *f*, *g* = vital staining of the anterior part of the primitive streak. *f* = immediately after staining. *g* = 24 hours later. *h n.* = Hensen's node. *h p* = head process. *n c* = notochord. *o v* = optic vesicle. *p s* = primitive streak. *Si* = first somite.

a square window, about 10–15 mm square in the uppermost part of the shell, using the hack saw blade. Carefully avoid any injury to the shell membrane. Lift the window out with the scalpel and watchmaker forceps.

6 Moisten the shell membrane thoroughly with saline solution, rupture and remove the shell membrane with the watchmaker forceps. Carefully avoid any injury of the blastoderm Remove excessive egg albumen with the pipette, if necessary Use sterile pipettes.

7 Usually the primitive streak does not stand out clearly To make it distinctly visible, place a rather large piece of blue agar on the central area of the blastoderm Do not remove the vitelline membrane. The stain diffuses rapidly through it. Remove the agar as soon as the streak becomes visible avoid deep staining Identify Hensen s node Select embryos in definitive primitive-streak stages

8 With the watchmaker forceps place a small piece of red agar, slightly larger than Hensen s node, in the desired position Stain deeply for several minutes (the time depends on the concentration of the dye in the agar) Moisten the blastoderm before you remove the red agar, then pick it up with the watchmaker forceps

9 Make a sketch of the embryo and of the mark It is necessary to have an exact drawing of the position of the mark, in order to be able to interpret the results.

10 Seal a cover glass over the window in the following way with a small brush apply hot melted paraffin to the edges of the window Place the cover glass over the window and press it gently Add paraffin if necessary

11 After 24 hours remove the cover glass enlarge the window, cut out the entire blastoderm with a fine forceps transfer it with an extra wide pipette to a watch glass with saline Study and draw the extent of the mark its shape etc. Handle the embryo with watchmaker forceps. Dissect the skin away if necessary Use a very strong light source.

Do 2–3 experiments of each type

c) VITAL STAINING OF HENSEN S NODE (FIG 33 *c–e*)

EXPERIMENT 42

Procedure

Stain Hensen s node following the procedure given on page 140 After 24 hours remove the blastoderm from the egg Slit the neural tube in the midline with a glass needle hold the embryo with a forceps or turn the

Some investigators recommend placing a small granule of neutral red powder on the area to be stained. Control carefully· avoid overstaining

blastoderm upside down Locate the stain in the notochord and ventral aspect of the neural tube Use high power and very bright illumination.

d) VITAL STAINING OF PART OF THE ANTERIOR HALF OF THE PRIMITIVE STREAK (FIG 33 *f–g*)

EXPERIMENT 43

Procedure

Vital-stain a small area of the primitive streak a short distance behind Hensen s node Make an exact sketch of the position and extent of the mark. After 24 hours, recover and dissect the embryo as before. Determine the number of somites which are stained Try to find the stain in the neural tube. Use a strong light source.

e) VITAL STAINING OF A PRENODAL AREA (FIG 33 *a b*)

EXPERIMENT 44

Procedure

Place the mark in front of Hensen s node, near it and to the right of the median plane. Make a precise sketch. After 24 hours dissect the embryo find the stain Notice which parts of the brain are stained

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2. CHORIO-ALLANTOIC GRAFTS

a) GENERAL REMARKS

Review the formation and structure of the chorio-allantoic membrane (see Fig 34, *a b*)

The main experimental methods in chick embryos are as in amphibians isolation extirpation and transplantation of embryonic areas Complete isolation can be accomplished by *in vitro* culture on a watch glass or a cover slip using embryonic extract as a nutrient substrate The *in vitro* technique is too difficult to be used in classroom experiments Those who are interested in it will find technical details in Waddington (1932) and Spratt (1940)

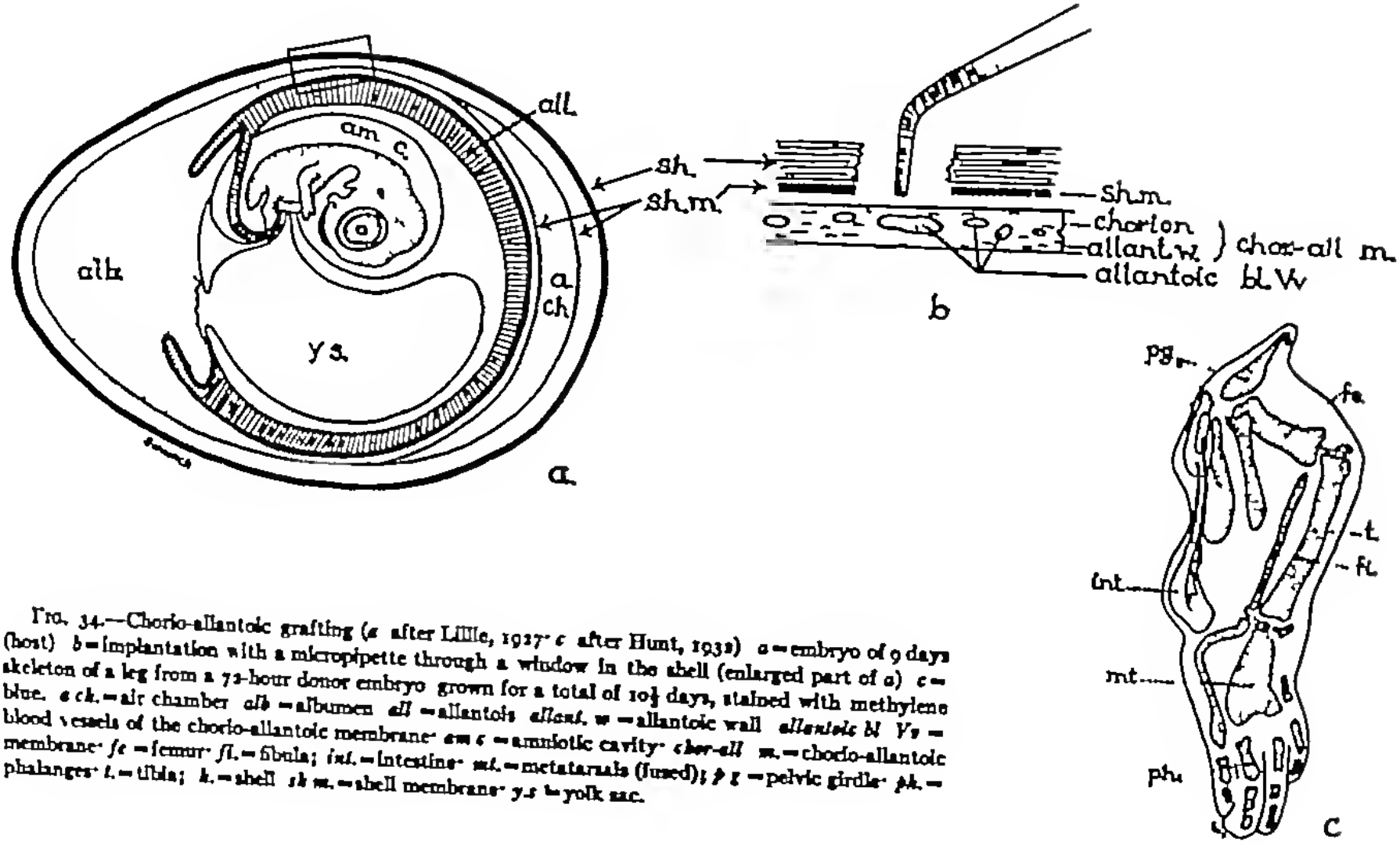

FIG. 34.—Chorio-allantoic grafting (*a* after Lillie, 1927; *c* after Hunt, 1932) *a* = embryo of 9 days (host) *b* = implantation with a micropipette through a window in the shell (enlarged part of *a*) *c* = skeleton of a leg from a 72-hour donor embryo grown for a total of 10½ days, stained with methylene blue. *a ch.* = air chamber *alb* = albumen *all* = allantois *allant. w* = allantoic wall *allantoic bl Vs* = blood vessels of the chorio-allantoic membrane; *am c* = amniotic cavity; *chor-all m.* = chorio-allantoic membrane; *fe* = femur; *fl.* = fibula; *int.* = intestine; *mt.* = metatarsals (fused); *p g* = pelvic girdle; *ph.* = phalanges; *t.* = tibia; *sh.* = shell *sh m.* = shell membrane; *y s* = yolk sac.

The technique of *chorio-allantoic grafting* is widely adopted as an isolation method. It was worked out in the present form in Dr F R. Lillie's laboratory at the University of Chicago by Hoadley (1924) and Willier (1924) It makes use of the highly vascularized chorio-allantoic membrane of avian embryos which becomes closely applied to the shell membrane and is thus within easy reach of the experimenter (Fig 34) The membrane of 8½–10-day chick embryos is sufficiently developed to serve as a substrate The structures whose potencies are to be tested are placed on the membrane through a window in the shell which is sealed up after the operation. The grafts become vascularized and can be reared for 9–10 days. They have to be recovered before the chorio-allantoic membrane breaks down on the nineteenth day of incubation

The transplants are completely isolated from the structures of the host embryo proper, so that inductive effects etc. cannot obscure the results. On the other hand, the transplants being incorporated into the blood circulation of the host, are exposed to all substances carried in the blood stream for instance the hormones In this respect the isolation is incomplete and the possible effects of these agents on the differentiation of the transplant must be taken into consideration This aspect has been made use of in the analysis of the influence of sex hormones on indifferent gonad primordia (see Willier 1939) Another limitation of the method lies in the inadequate space which the transplant finds on the membrane It flattens and is buried in membrane cells Therefore one never obtains normally shaped eyes or limbs In evaluating the results one must be cautious not to attribute such failures to the lack of inherent potencies of the transplanted primordia

A promising new field was opened when it was found that mammalian tissue can be grown successfully on the chick membrane (see Nicholas and Rudnick 1933) Recently the membrane was found to be a suitable substrate for a large number of viruses and of bacteria including forms pathogenic for man and forms which are difficult to culture otherwise (review in Goodpasture 1938)

b) CHORIO-ALLANTOIC GRAFTS OF LIMB PRIMORDIA

The experiment demonstrates that limb structures are capable of self differentiation when isolated from the body at 2–3 days of incubation This experiment was made first by Murray and Huxley (1925) and by Murray (1926) This author grafted whole limb buds and proximal distal or longitudinal halves from 3–5-day embryos and found a rather rigid mosaic development of fragments For instance the proximal part

of a leg bud from a 3-day donor formed femur, and the distal part formed the tibiotarsus the fibula, and foot The cartilages are usually abnormal in shape and the skeletons incomplete owing to unfavorable conditions on the membrane The differentiation of the limb musculature on the membrane was studied by E A Hunt (1932) The percentage of well developed transplants is usually small. Figure 34, *c*, shows an exceptionally well-differentiated graft (from Hunt, 1932) It is advisable to make 4–6 operations at a time

EXPERIMENT 45

Material

10–12 eggs per student
standard equipment (p. 133)

Procedure

1 *Incubation of the hosts* —Start the incubation of the hosts (5–6 per student) 9 or 10 days before the operation day Mark the date of incubation, or an *H*, on each egg Turn the eggs once or twice daily

2 *Incubation of the donors* —Start the incubation of the donors (5–6 per student) 65–70 hours before the laboratory period Mark the date of incubation, or a *D* on each egg Roll the eggs once or twice a day

3 Autoclave the material marked with an asterisk (p 134) several hours before the beginning of the laboratory

4 *Other preparations to be made in advance* —Begin the heating of the warming plate and of the paraffin 1 hour before operations. Have the candles ready Place donors and hosts in separate trays mark all trays clearly Have empty wire trays (hatching trays) ready for the temporary placement of hosts in the nest and have empty turning trays ready for the hosts after operation

5 *Preparations to be made by each student* —Place forceps scalpels, scissors and hack-saw blade in 70 per cent alcohol, 1 hour before the operations start Prepare two nests by placing a padding of cotton on watch glasses. Mold the cotton into a shallow groove into which the egg will fit Fill 2 watch glasses with sterile saline solution and cover them with a lid Keep the third watch glass dry and covered Immediately before operation cover the operation table with sterile towels Dry all steel instruments carefully Place them and all pipettes in a fold of a sterile towel and place the towel to the right of the binocular microscope Wash your hands

Note —Follow two rules Work as fast as you can but not hastily Keep every tool as sterile as possible

PREPARATION OF THE HOST EMBRYO

6 Candle host eggs Discard those which are sterile and those with dead embryos [3] Living embryos show clearly the chorio-allantoic vessels and indistinctly the embryo as a dark area. Its rocking movements may be seen in candling

7 Select and mark a suitable site for implantation. Over the candle locate the main blood vessels of the chorio-allantoic membrane Choose a point of junction of two strong vessels at some distance from the embryo Mark this point with a pencil on the shell.

8 Saw the window It should be small to reduce the chance of infection. Place the egg on a nest marked side up Saw a rectangular hole about ½–1 cm Saw only 3 sides The fourth side will break when the window is lifted up Saw slowly and steadily without jerks Be sure to avoid sawing through the shell membrane The chorio-allantoic membrane is closely applied to the latter and its injury results in a hemorrhage Before you remove the window draw a pencil line across one edge of the window so that you can fit it in again in the right orientation Lift the window out with the scalpel and place it in the dry watch glass Cover the latter with a lid

9 Moisten the shell membrane thoroughly with saline solution using a small pipette. This is absolutely necessary because otherwise the shell membrane cannot be removed without injury to the chorio allantoic membrane. Under the low power of the binocular microscope pick up the shell membrane cautiously using the watchmaker forceps rupture and remove it or sever it on three sides only The junction of the blood vessels should now be visible.

10 The host is now prepared. Place the shell piece back in the window Mark the egg with your initials and place it on its nest on a wire tray in the incubator until the transplant is prepared.

PREPARATION OF THE TRANSPLANT

11 Candle the donor embryo and mark its position Place it on a cotton nest and saw a large square window around the marked region. No precautions are necessary the shell may crack and the vitelline vessels may rupture

12 Moisten the shell membrane over the embryo and remove it with the watchmaker forceps The embryo will now be exposed

13 With a pair of fine scissors cut out the blastoderm a short distance from the embryo Transfer it quickly to a watch glass containing warm saline solution, using the wide-mouthed pipette or pick it up with a fine forceps Discard the egg

[3] This is best done by the instructor before the laboratory period

14 The embryo is covered with amnion and chorion Rupture and remove these very carefully with 2 watchmaker forceps. Use one to hold the embryo and the other to remove the membranes Work under the low power of the binocular microscope against a dark background

15 Locate the wing and the leg buds Before isolating them, first make two transverse cuts through the entire embryo, one through the neck one between wing and leg buds (*x*, *y* in Fig 35, *a*) Use a forceps for holding the embryo and an iris knife (Fig 1, *f*) for the cutting Next, dissect out one bud by making four cuts in the sequence 1, 2 3, 4 or 5 6 7 8 (Fig 35, *a*) Do not cut too close to the bud rather include parts of the somites Remove fragments of yolk and blastoderm adhering to the bud When the transplant is ready for grafting cover the watch glass and put it on a beating plate

TRANSPLANTATION

16 Take the host out of the incubator and remove the shell piece from the window, placing it in a covered sterilized watch glass.

17 Suck the transplant into the micropipette with a small amount of saline Introduce the tip of the micropipette into the window (Fig 34 *b*) and drop the transplant onto the chorio-allantoic membrane by applying gentle pressure on the rubber membrane of the pipette. Operate under the binocular microscope and try to place the graft near the blood vessel. Observe carefully to see if the transplant is actually on the membrane. It sometimes sticks to the pipette If this happens then pipette it back into the watch glass with strong squirts and repeat the procedure.

18 Fit the piece of shell back and seal it in with hot paraffin With the paraffin brush go over all four edges. Give the egg a protocol number Return the host to the incubator, with the window facing downward

19 Take a careful protocol indicate the exact age of donor and host. Note whether it was a wing or leg bud etc.

Repeat the experiment using both wing and leg buds If the material is scarce several buds from the same donor should be taken It is also possible but not advisable to implant 2 grafts in the same host

RECOVERY OF THE GRAFT

Materials

several finger bowls
several liters of warm saline (NaCl 0 9 per cent 38°C)
a pair of strong scissors and a pair of fine scissors
watchmaker forceps
Bouin s fixative
small vials with cork stoppers
labels
a jar with etherized cotton for discarded host embryos

20 The graft should be taken out when the host embryo is 18–19 days old i.e. 9–10 days after operation. If the graft has taken it will be found under the window Make a circular cut through shell and chorio-allantoic membrane at a radius of about 1–2 cm from the window Wash this piece of shell in the finger bowl and inspect it from the inner side The chorio-allantoic membrane covers the inside of the shell, and the graft will be seen under the window as a pink nodule of varying size sometimes with fluid filled vesicles attached to it. Limb structures are rarely recognizable the transplant must be cleared and stained to make them visible Cut the graft out very carefully using fine scissors and transfer it to a vial half filled with Bouin s fixing fluid Label the vial with your initials and the case number Enter your findings in the protocol. Place the host embryo in the jar with etherized cotton and discard it later on.

21 *Staining and clearing of the graft* —The Lundvall technique of staining cartilage is recommended for a quick preparation of the skeleton of the grafts for further study (see p 135 and Fig 34 *c*) Students who are interested in the differentiation of the musculature etc. should section and stain their grafts

22 *Make protocol sketches of the stained grafts* also of the best specimens obtained by other members of the class. Compare with the figures in Murray (1926) and E A Hunt (1932)

c) CHORIO-ALLANTOIC GRAFTS OF EYE PRIMORDIA

EXPERIMENT 46

The optic vesicle of a 33 hour embryo will undergo considerable differentiation when isolated and grown on the chorio-allantoic membrane Retina, pigment epithelium and lens may be distinguished in the graft However there is a high degree of variation in the shape of the transplants most of them will be small solid nodules with abnormal arrangement of the different layers and only a few will be vesicular structures approaching a normal eye (Hoadley 1924 Alexander 1937) These abnormalities are due to unfavorable conditions on the membrane since primordia of the same stage proceed much further toward normalcy when grafted to the flank or into the coelom

Material

the same as in Experiment 45

Procedure

The method is the same as in Experiment 45 except for the following changes

2 (p 146) Incubate the donors for about 33 hours to obtain embryos of about 9–12 somites

15 (p 148) Amputate the head in the hind brain region Cut out the right optic vesicle and forebrain follow the dotted lines in Figure 36 *b* Remove the underlying blastoderm The forebrain and epidermis with lens epithelium are included in order to make the graft bulkier

21 (p 149) Clearing in oil of wintergreen or xylol will bring out the main structures, such as pigment epithelium and lens. Staining is not necessary The grafts must be sectioned if histological studies are desired.

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3 INTRA EMBRYONIC TRANSPLANTATIONS

a) COELOM GRAFTS OF LIMB PRIMORDIA
(After Hamburger 1938 1939)

The coelomic cavity of the chick embryo is an especially favorable site for the transplantation of primordia It has certain advantages over the chorio-allantoic membrane it allows for undisturbed expansion and nor

mal morphogenesis of such primordia as limb buds and optic vesicles Furthermore it gives the transplant a longer life span since 3-day embryos are being used as hosts The transplants are slipped through a hole in the somatopleure and attach themselves to the coelomic walls mesenteries or to parts of the umbilical cord and receive blood supply from the host Since transplants which develop at a distance from the central nervous system have little chance to receive a nerve supply it is possible in this way to obtain noninnervated organs for instance completely nerveless limbs which are paralyzed from the start They were found to be remarkably normal, particularly with respect to their skeleton and their joints (Hamburger 1939 Hamburger and Waugh 1940) Even the musculature will undergo normal differentiation and cross-striation but innervation is required for its maintenance and without this it degenerates *shortly after its initial differentiation* These cases demonstrate that innervation and functional activity during development play only a minor role as causal factors in limb morphogenesis Other primordia have been reared successfully in the coelomic cavity for instance optic vesicles (Joy 1939) spinal cord (Bueker unpublished) and mouse tissue (Rawles 1940 Gluecksohn Schoenheimer 1941)

EXPERIMENT 47

Material

3–4 donors and 3–4 hosts per student
standard equipment (p 133)

Procedure

1 Start the incubation of both donors and hosts about 2½ 3 days before the operation For your particular incubator find out the time required to reach stages 3–5 (29–38 somites see p 133)

2 Autoclave all material marked with an asterisk (*) on page 134 starting several hours before the operations

3 Make other preparations as in sections 4 and 5 on page 146

4. Cut out small pieces of red agar for vital staining and place them in sterile saline solution Their size can best be determined when the first embryo is stained It should be large enough to cover the right half of the embryo posterior to the tenth somite Prepare half of a dozen agar pieces or more They must be replaced by fresh pieces when they begin to fade

VITAL STAINING OF THE HOST EMBRYO

5 Candle the egg If the embryo is stuck on the side it is often possible to bring it into the desired position on top by gentle shaking or rolling With pencil mark a square of about 1½ cm edge on the shell above the embryo

6 Place the egg on a nest (cotton pad on watch glass), marked side up, and saw a square window as marked. Very carefully avoid sawing through the shell membrane and thus injuring the embryo Hemorrhages are usually fatal Saw slowly and steadily Saw only 3 sides and break the fourth side when the window is lifted up with the scalpel If the shell piece is to be sealed back place it in a sterile, dry dish Avoid the loosening of small shell particles on the edges of the window

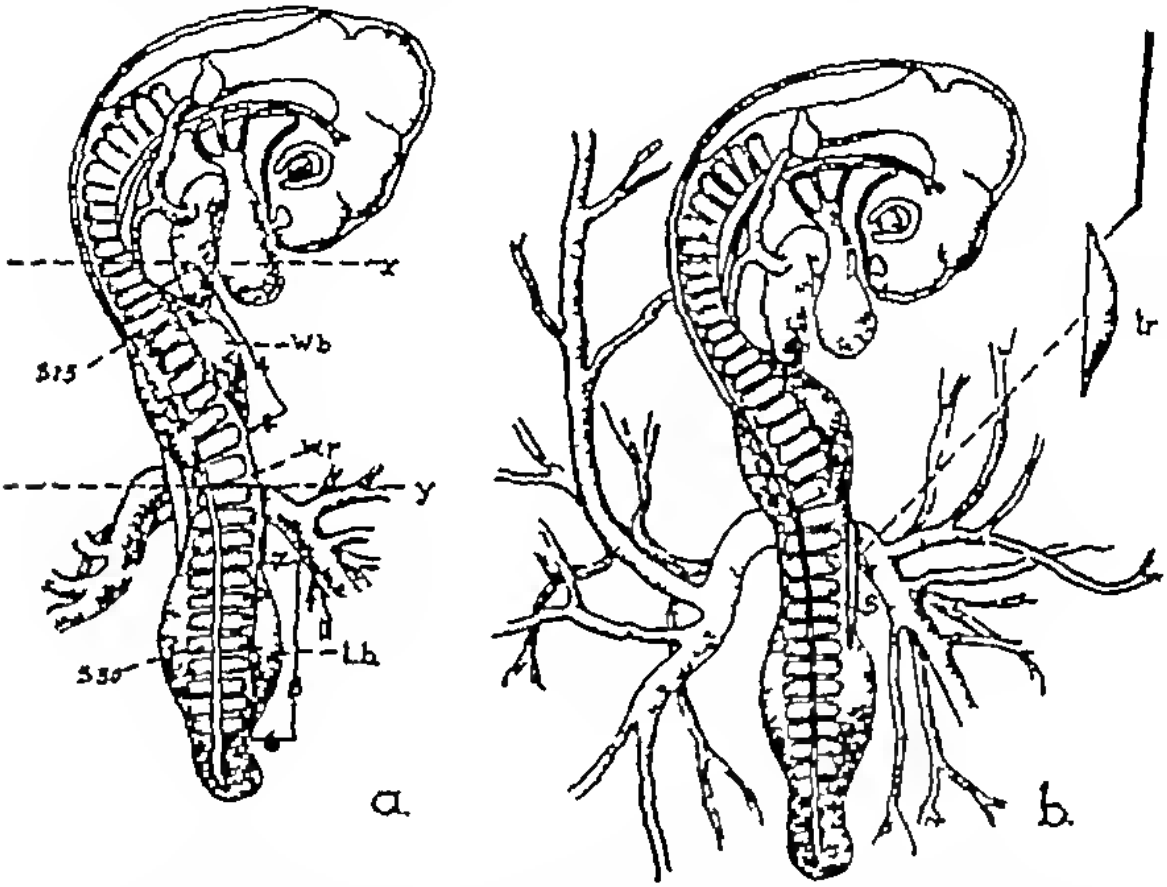

FIG 35 —Limb-bud transplantation in the chick embryo. *a* = donor embryo *b* = host embryo (both 34 somites, 72 hours of incubation) *Lb* = leg bud *s* = slit for the reception of the transplant *S15 S30* = somites 15 30 *tr* = transplant, *w b* = wing bud *w r* = Wolffian ridge for other letters and numbers see text.

7 Moisten the shell membrane thoroughly with saline solution otherwise it cannot be removed without hemorrhages Under the binocular dissecting microscope remove it very cautiously in the area of the window using a pair of watchmaker forceps The embryo should now be exposed If it sticks on one side roll or shake the egg gently and try to move it to the top Add sufficient sterile salt solution to keep it moist The embryo should settle rather deep under the shell

8 For vital staining place a piece of red agar over the right flank covering the right wing bud and the region posterior to it. Cover the window

with the shell piece or with a cover glass Mark the egg with your initials and with a serial number and return it to the incubator for 5–10 minutes

Note—If the agar piece is deep red and rather thick then it is best to stain through the vitelline membrane The latter stains first but will soon give off all dye to the adjacent tissues We prefer thin agar films Rupture the vitelline membrane with the watchmaker forceps before staining then place the agar directly on the embryo and thus obtain a deep stain within a short time.

PREPARATION OF THE TRANSPLANT

9 Candle another egg marking the embryo as before Place the egg on a nest and break or saw a large hole considerably wider than that in the host so that the embryo and the adjacent area vasculosa are laid open. With a pair of (sterilized) scissors cut out the entire area vasculosa with the embryo in its center With the watchmaker forceps or with a wide-mouthed pipette transfer it to a dish with saline solution

10 Under the binocular microscope turn the blastoderm right side up flatten it and hold it with the left forceps Use a dark background Locate wing and leg buds Count the somites determine the stage (p 133) and protocol these data Amputate the head with a Knapp iris knife (level *x* in Fig 35 *a*) and discard it Hold the embryo with the left forceps and cut out the right wing or leg bud with the iris knife Make four cuts the first longitudinal and median to the bud close to the somites the second and third perpendicular to this in front of and behind the bud and the fourth lateral and not too close to the bud (1 2 3 4 or 5 6 7 8 in Fig 35 *a*) The entoderm may be peeled off using 2 forceps but this is a rather delicate procedure and is not necessary When the transplant is isolated cover the dish with a lid and place it on a heating plate (not warmer than 39° C)

PREPARATION OF THE SITE OF IMPLANTATION IN THE HOST EMBRYO

11 Reopen the host embryo add saline solution and under the binocular microscope remove the agar with a pair of watchmaker forceps Shake the embryo gently if the blastoderm adheres to the edge of the window

12 If the amnion and chorion cover the operation region it is necessary to slit them open This is done with a glass needle Hold the egg shell with your left hand insert the needle into the amniotic cavity in the midline where the raphe (suture) is visible and with a jerky upward movement of the needle rupture the membranes Continue this until at least the posterior half of the right wing bud is exposed The membranes require no further care They will heal back over the embryo

13 The transplant is to be implanted through a hole posterior to the wing bud (*s* in Fig 35, *b*) Locate wing and leg buds and vitelline arteries In the following steps it is absolutely necessary to avoid hemorrhages. Be sure not to get too close to the lateral edges of the somites, to avoid puncturing of the posterior cardinal vein, which runs underneath the lateral edges of the somites Do not push the needle so deeply as to injure the splanchnic layer which is highly vascularized Under high power of the binocular microscope push the glass needle through the somatopleure at a point between the wing and the leg bud and a short distance lateral to the outer edges of the somites which stand out clearly in red From this hole work forward and backward and make a longitudinal slit in the somatopleure parallel to the main axis and large enough to allow the passage of a limb bud Add saline to keep the embryo moist

TRANSPLANTATION

14 Under the binocular microscope suck the prepared limb bud into the distal part of a micropipette By gentle pressure on the rubber membrane which covers the lateral hole drop the transplant onto the host near the slit. This should be done under the binocular microscope (lowest power) to avoid the loss of the transplant. Hold the egg shell with your left hand and manipulate the transplant through the slit into the coelom using the tip of the glass needle (Fig 35 *b*) The transplant may be oriented during or after the implantation If one wishes to have the transplant adhere to the umbilical cord, it has to be pushed into a lateral position at a considerable distance from the somites Otherwise leave it near the somites above the root of the vitelline artery In longitudinal orientation Add a small amount of saline solution Take a complete protocol note if wing or leg has been transplanted orientation stage of host etc.

15 Seal the window Place either the original piece of shell which was sawed out or a square or circular cover glass over the window With a brush apply warm paraffin around the edges Be sure that the window is sealed completely on all sides Return the egg to the incubator Place it on a strip of cotton the window facing upward

RECOVERY OF THE TRANSPLANT

16 Allow the host to develop for 7–9 days the best stage for fixation is 10–12 days of incubation Do not roll the eggs during this period Prepare a pan or finger bowl with warm saline solution Remove the window and widen the hole Carefully dissect away the chorio-allantoic membrane Carefully sever the umbilical cord and lift the embryo into the dish of saline If no transplant is visible from the outside it may be entirely hidden inside of the coelomic cavity or it may have been resorbed

Carefully slit open the ventral body wall slightly to the left (apparent right) of the median line and inspect the inside of the body cavity Take careful protocols Fix the host embryo together with the transplant in Bouin and stain them with methylene blue (p 135) to make the host and the transplant skeleton visible

b) FLANK GRAFTS OF LIMB PRIMORDIA

(After Hamburger 1938 1939)

The method of implantation into the coelomic cavity has the disadvantage that the transplants cannot always be fixed in a given position. If a definite orientation is desired the primordia are best implanted in the outer body wall

EXPERIMENT 48

Material

2–3 donors and 3–4 hosts per student
standard equipment (p 133)

Procedure

As in Experiment 47 with the following modifications

10 (p 153) Preparation of the transplant In cutting out the limb bud leave small strips of tissue (somites or adjacent mesoderm) attached to the anterior and posterior ends of the base of the bud these will be tucked into the slit. The other limb buds may be used for further transplantations

13 (p 154) Preparation of the slit in the host embryo Make the slit between wing and leg bud as close to the somites as possible and not too long Rather lengthen it while you implant

14 (p 154) Implantation With the tip of the glass needle first tuck the anterior then the posterior end of the limb bud into the slit (see Fig 35, *b*) If the hole is slightly shorter than the bud the transplant will be held in position by the tension of the tissues Take care that the major part of the transplant is exposed and that it does not slip into the coelom No other precaution is necessary to keep the transplant in position

15 (p 154) In handling the egg after the operation be exceedingly cautious avoid all sudden or jerky movements Place it on cotton in the incubator and do not disturb it for a day or two

c) FLANK GRAFTS OF EYE PRIMORDIA

(After Gayer 1942)

The first flank grafts of optic vesicles were made by Alexander (1937) in connection with the problem of lens induction Gayer (194) obtained

well formed eyes by the same method, using optic vesicles of 10–20-somite stages The shape and general structures of the transplants were normal This demonstrates the high degree of self-differentiating capacity of the optic vesicle with respect to its surrounding structures. However, practically all flank grafts showed a deficiency in the closure of the choroid fissure of the type which is occasionally found as a congenital abnormality in human eyes and which is known in ophthalmology as "coloboma."

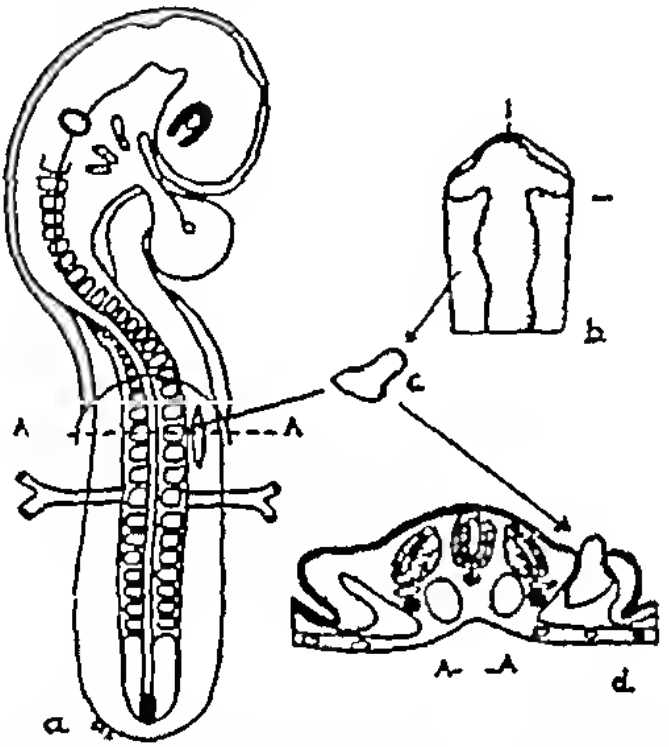

FIG. 36 —Transplantation of an optic vesicle in the chick (after Gayer 1942) *a* = host embryo (30 somites, 60 hours of incubation) with slit for the implantation of the optic vesicle *b* = head of the donor embryo (12 somites, 36 hours of incubation) the dotted lines indicate the cuts by which the right optic vesicle and the adjacent brain part are severed *c* = transplant *d* = cross-section through *a*, in level *A–A* showing the transplant in position.

EXPERIMENT 49

Material

2–3 donors 3–4 hosts per student
standard equipment (p 133)

Procedure

As in Experiment 47 with the following modifications

1 (p 151) Start the incubation of the *host* embryos 2½–3 days and the incubation of the *donors* 36–42 hours before operation At operation the donor should have 10–15 somites The host should be in stages 3–5 (p 133)

8 (p 152) Vital stain the right wing level rather than more posterior parts of the host.

10 (p 153) Cut out the right optic vesicle together with the right half of the forebrain. First, make a transverse cut behind the right optic vesicle through the head to the midline Second make a median cut through the anterior part of the head. The left eye may be used for another transplantation (Fig 36 *b*)

13 (p 154) Make the slit at the base of the wing at about the level of the twentieth somite (Fig 36 *a*)

14 (p 154) Transfer the optic vesicle with adhering brain tissue onto the host blastoderm, using the micropipette. Drop it near the slit With the tip of the glass needle tuck the brain portion into the slit, thus leaving the optic vesicle exposed on the surface (Fig 36 *d*)

16 (p 154) Incubate the host for not more than 9–10½ days (total age) i.e. 7–8 days after operation at which stage all essential eye structures are differentiated In order to make visible such details as lens retina iris choroid fissure etc. without sectioning the transplant the embryo may be fixed in Bouin dehydrated and cleared in oil of wintergreen (p 135)

d) TRANSPLANTATION OF NEURAL CREST FROM DARK TO WHITE BREEDS TO DEMONSTRATE THE ORIGIN AND MIGRATION OF MELANOPHORES

(After Willier and Rawles 1938 1940)

The dark pigment of vertebrates is contained in granular form in pigment cells which are called 'melanophores They originate in the neural crest. This has been convincingly demonstrated for amphibians and birds by extirpation transplantation, and tissue-culture experiments (reviews in Harrison 1938 Willier and Rawles 1940) The potential melanophore cells, like other neural-crest cells are wandering cells They migrate from the dorsal part of the neural tube to their final locations where they proliferate and form complex color patterns for example in the skin of amphibians or in the feathers of birds. In the latter instance they migrate into the developing feather germs and deposit pigment granules in the barb primordia. The migration of melanophores can be demonstrated by the following experiment devised by Willier and Rawles (1938 1940) A small piece of embryonic head epidermis from a dark breed of fowl including some prospective melanophores was transplanted to the base of the wing bud of a 2–3-day embryo of a white breed The transplanted epidermis was not incorporated in the epidermis of the host, but the prospective melanophores adhering to it migrated into host territory

They settled down in the developing feather germs of the host wing and there deposited their pigment As a result the fully developed wing was partly or entirely covered with black down feathers, all other feathers being uncolored In some cases the pigmented area extended as far as to the ventral midline of the body The melanophores thus exhibited not only an extensive migration but an extraordinary proliferative capacity they all originated from the few neural-crest cells which adhered to the epidermis transplant.

Willier and Rawles and their associates carried the experiment further The hosts were allowed to hatch In due time the down feathers were replaced by the adult plumage White Leghorn wings which carried a transplant of Barred Rock melanophores exhibited a typical barred pattern in their feathers These experiments reveal the unexpected fact that the color pattern is largely determined by the genetic constitution of the melanophores rather than by that of the feathers themselves However the host feather germs modify and control the activity of the melanophores to a certain extent. The role of both partners in the determination of the final pattern was analyzed by means of extensive series of reciprocal transplantations between many different breeds of fowl (review in Willier 1941)

EXPERIMENT 50

Material

donors any dark breed e g , Brown Leghorns 2–3 eggs per student
hosts 3–4 eggs, White Leghorn
standard equipment (p 133)

Procedure

1 Start the incubation of the donors and hosts 2½–3 days before operation. However younger donors of 30–40 hours of incubation (5–10 somites) may be used (Dorris, 1939) The hosts should not be older than stage 4 (p 133)

2–7 As in Experiment 47 (p 151)

8 Prepare the slit in the host. Rupture chorion and amnion over the right wing bud open up a large hole The membranes will heal over again With the glass needle make a small but rather deep hole at the base of the wing Vital staining is not necessary Return the host to the incubator

9 Vital stain the head skin on the donor Place a piece of red agar on the head in front of the otocyst. Stain for 5–10 minutes.

10 Meanwhile take the host out of the incubator, place it within easy reach When the skin of the donor is sufficiently stained cut out the

transplant as follows With the glass needle or watchmaker forceps strip a piece of skin from the dorsal and dorsolateral surface of the head in front of the otocysts This piece contains neural crest cells

11 Transfer this piece directly onto the host using the micropipette

12 Implant the graft in the prepared slit bury it deeply Be sure that the transplant sticks to the slit

13 Seal the window and return the egg to the incubator the window facing upward Mark the egg with your initials and a protocol number

14 Recover the host about 2 weeks after the operation Pigment has formed at that stage Study the color of the wing the extent of the pigmented area etc. (see papers quoted above)

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PART IV

REGENERATION (RECONSTITUTION)

A GENERAL REMARKS

The regenerative potencies vary greatly among animals *Hydra* and other Coelenterates the planarians among the Platyhelminthes and the *Urodela* among the vertebrates are known for their extraordinary regenerative power A survey of the regenerative properties of the different animal groups may be found in Korschelt (1927) see also Morgan (1901) and Child (1941)

It has long been recognized that the repair of lost parts can be accomplished in two ways by internal reorganization and transformation of old tissues without addition of new growth or by outgrowth of new tissue from the cut surface in the form of a regeneration bud or blastema Head and tail regeneration in *Planaria* limb and tail regeneration in *Urodela* are examples of the latter type The change in shape as well as the formation of a new pharynx by the old tissue in the regeneration of *Planaria* is an example of the former type. In many instances both types are combined in the same form and there may be no fundamental difference between them but for practical purposes it is desirable to designate them with different technical terms Morgan (1901) distinguishes between 'epimorphosis' (proliferation of new tissue) and morphallaxis (changes within the old tissue) and includes both under the general heading regeneration Child (1941 p 30) uses the term reconstitution to include all types and defines regeneration in the narrower sense as reconstitution by outgrowth and reorganization as reconstitution by internal changes

Several experiments described below illustrate the fact that precisely those parts which were removed are regenerated This is to be expected from the teleological point of view but the underlying causal factors which must be different at different levels of the regenerating organ are not clearly understood Cases in which more or less tissue is regenerated than was removed and cases in which the regenerated structure is different from the lost part (heteromorphosis) are of particular interest in this connection These points will be illustrated below

The young regeneration blastema with its underlying stump have all characteristics of a morphogenetic field (p 94) The organ forming properties of the blastema are at first labilely determined and gradually become fixed This was shown by transplantation experiments (see Schotté 1939

Weiss, 1939) The blastema passes through a stage in which it is determined as a whole but not as a mosaic of details Duplications arising from single blastemas bear out this point. Regeneration fields,' like embryonic fields extend beyond the boundaries of the regenerating organs. For instance, if a urodele limb is extirpated with its girdle, tissue adjacent to it will regenerate a complete limb However, the limb-regeneration field, as well as other regeneration fields do have definite limits, and no regeneration occurs if the entire field is extirpated In this sense regeneration is not a property of the whole organism but of a local field P Weiss (1925, etc.) and Guyénot (1927, Guyénot and Ponse, 1930) have developed the concept of regeneration fields

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B REGENERATION IN PLANARIA

1 LIVING MATERIAL CULTURE METHODS

Two native species are commonly used for experiments *Dugesia ti grina* Girard (syn *Planaria maculata Euplanaria tigrina* Leidy), and *D dorotocephala* Girard (syn *Pl dorotocephala* Woodworth *E dorotoceph ala* Woodworth) They can be distinguished readily as follows

Dugesia dorotocephala is larger than *tigrina* (length up to 25 mm) it is uniformly dark (brown or black) its auricles are elongated and pointed (Fig 37 *a*) it lives in springs and spring fed streams and can be collected by baiting It is found in middle western states

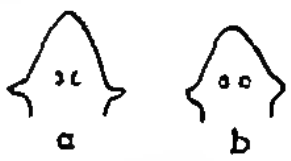

FIG 37 —*a* = head of *Dugesia* (*Pla aria*) *dorotocephala* *b* = head of *D tigrina* (*Pl maculata*) (from Hyman 1931)

Dugesia tigrina is, at best 15–18 mm long it is variable in color usually it shows a spotted color pattern (white irregular spots on a brownish or blackish background) or a light mid-dorsal stripe. Its auricles are broader and blunter than those of *D dorotocephala* (Fig 37 *b*) It lives in ponds, lakes and slow flowing streams on the under surface of stones and leaves and can be collected by turning these over Its distribution is eastern and middle western states west to the Mississippi south to the Carolinas

Culture of planarians —Planarians collected in the field or bought from a dealer may be kept in large glass containers or dark enamel dishpans They should be covered and kept in a dark cool place Spring or well water is preferable to tap water Planarians should be fed twice a week with strips of calf or beef liver Before feeding lower the water to a depth of a few inches Distribute the strips of meat and remove them after 2 3 hours of feeding Thereafter rinse the dish or pan thoroughly and fill it with fresh water The animals are very susceptible to fouling of water (Most of these data are taken from Hyman 193, where more details may be found)

2. GENERAL EXPERIMENTAL PROCEDURE

Since the eighteenth century *Planaria* has been one of the favorite materials for the study of regeneration A systematic analysis was begun

Concerning the priority of the genus name *Dugesia* see Hyman (1939)

by Morgan (1898, 1900) Later on, Child and his associates used *Planaria* extensively for reconstitution experiments in connection with the gradient theory The voluminous literature on the subject is reviewed in Morgan (1901), Korschelt (1927), and Child (1941, and previous books) A large number of cutting experiments can be done as class experiments In the following only a few experiments were selected Others may be taken from the literature. Experiments which have a special bearing on the gradient theory will be found on page 189

Material for Experiments 51–58

Dugesia dorotocephala or *D tigrina*, about 12 specimens per student and per experiment (Species differences exist with respect to regenerative power, time of regeneration, etc. Since the speed of regeneration varies with temperature, it is desirable to run all experiments at constant temperatures)

finger bowls or Petri dishes
microscope slides
Planaria knife (p 9)
small brush to transfer planarians
pipettes

General procedure for Experiments 51–58

Note —Do not feed experimental animals for a week before operations For each experiment select 8 or 10 specimens of as uniform size as possible. Make all operations under the low power of the binocular microscope

1 Prepare and label finger bowls or Petri dishes one for 8–10 specimens Prepare a dish for discarded pieces

2 With a brush place a specimen in a drop of water on a clean slide Allow the animal to expand maximally Then make a cut with the Planaria knife in the desired plane. The knife must cut down in a perpendicular direction not obliquely The cut surface must be sharp and clean Practice on a few animals before you perform the protocoled experiments

3 Take a protocol and make a sketch

4 Transfer the piece which is to regenerate into the labeled dish and discard the rest of the animal at once

Note —Do not feed regenerating animals. Keep them in a cool dark place or cover the dishes containing regenerating animals with an inverted cardboard box.

5 Inspect the regenerating pieces every second or third day depending on the type of experiment Discard all dead pieces *Indicate clearly* on sketches the border line of old and new tissue Over a long period the regenerating tissue can be distinguished very clearly from the old tissue

by its lack of pigmentation Pay special attention to the first appearance of regenerating eye spots and pharynx Under ordinary conditions regeneration will be complete in about 8–14 days

3 REGENERATION AFTER TRANSVERSE LONGITUDINAL AND OBLIQUE CUTTING

Experiment 51 Regeneration of Transverse Pieces

Follow the directions on page 166 First decapitate the animal by cutting in plane *a* (Fig 38 *a*) Next make section *c* which is at one third of the length of the decapitated animal Remove the pharynx if it protrudes Place each piece in a separate dish label the dish Repeat the experiment on 6 animals Place all identical pieces in the same dish Make careful observations and sketches of representative cases Pay special attention to the following points wound healing the appearance of the unpigmented regeneration blastemas The head which is characterized by the eye spots is the first differentiated structure to appear in posterior pieces It is always formed by blastema cells Observe the first appearance of the pharynx It appears near the posterior cut surface in the anterior pieces and near the anterior cut surface in the posterior pieces usually being formed by old tissue Note the time required for completion of the regeneration The end result is in each instance a smaller but proportionate individual The original polarity is maintained in all fragments. Any level is capable of forming a head

Experiment 52 Regeneration of Short Transverse Pieces

Section behind the head (Fig 38 *a* level *a*) and bisect the body (level *d*) Cut the anterior half in 3 (one sixth) or 4 (one-eighth) pieces of equal lengths and keep all pieces of the same level in one dish Label the dishes Operate 6 animals In this experiment study mainly the attainment of the typical proportions by the formation of blastemas as well as by morphallaxis All short pieces are at the beginning much too wide as compared to their lengths The heads (old or regenerated) will at first be disproportionately large Note the gradual adjustments of the proportions by changes in shape Note again the regeneration of a new pharynx, usually in the old tissue The head malformations which occur frequently in short transverse pieces are discussed on page 187 (see Fig 42)

Section *c* is recommended because this cut will serve as a control for Experiment 64 If this experiment is not planned, then make section *d* (instead of *c*) which cuts the decapitated animal in half.

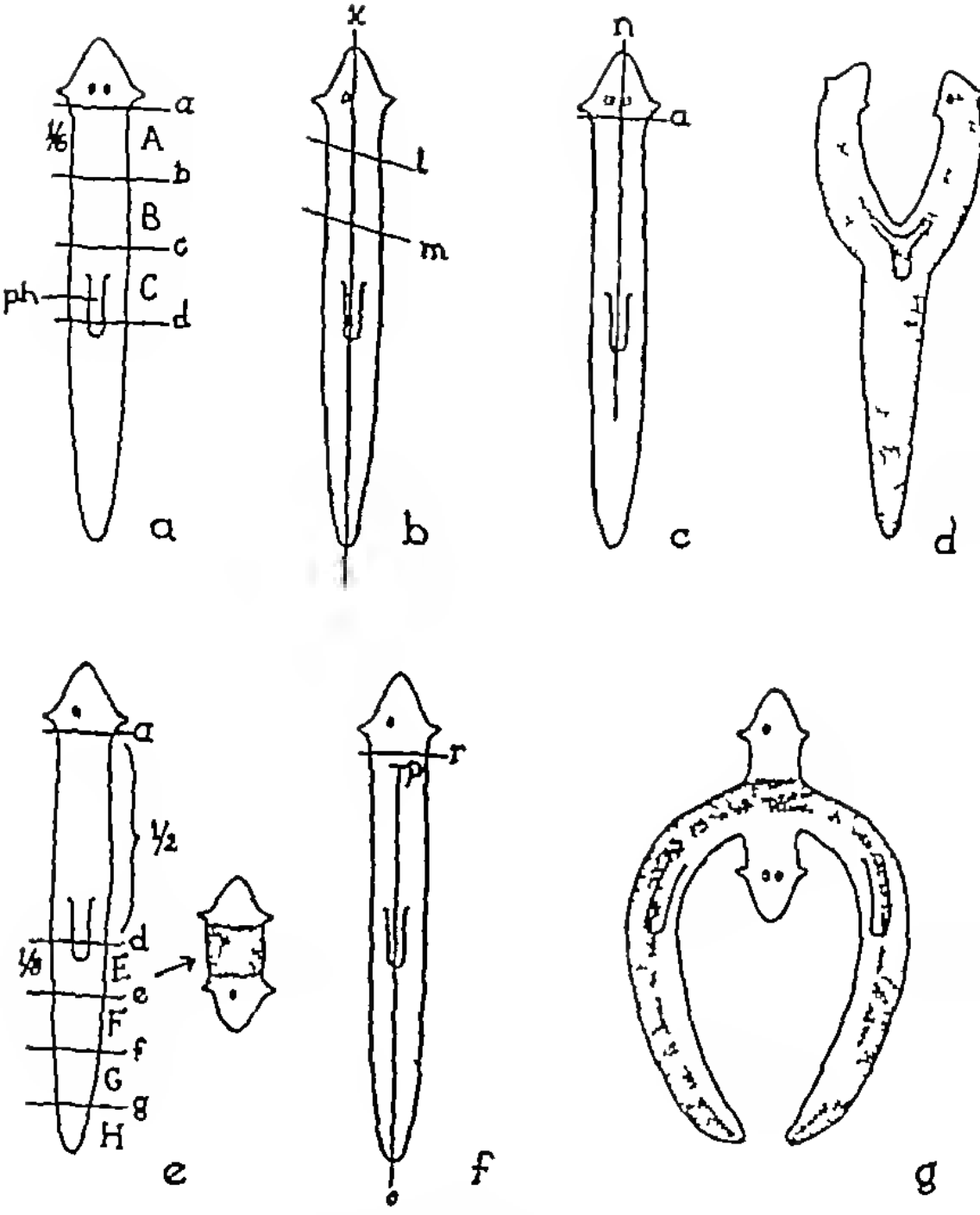

FIG 38.—Regeneration in planarians (*a* from Miller 1937 *f g* after Silber and Hamburger 1939) *a* = different levels of transverse sections *ph* = pharynx *b* = median (*b*) and oblique sections (*l m*) *c d* = production of duplicitas anterior by sectioning in levels *a* and *c* *e* = production of bipolar forms by regeneration of *E*-pieces *f g* = production of duplicitas cruciata by sections *e f* and *r* The dotted areas in *d e* and *g* are old tissue the light areas in these figures are regenerated tissue.

Section several planarians in the median plane (*k* Fig 38 *b*) Section others in a paramedian plane Discard all pieces of the old pharynx. Observe lateral regeneration

EXPERIMENT 54 REGENERATION FROM OBLIQUE SURFACES

Make an oblique cut and discard the anterior piece. In another series make two parallel oblique cuts in the prepharyngeal region (*l* and *m* Fig 38 *b*) discard head and posterior end Note that the head regenerating at the anterior surface makes its appearance not in the middle of the cut surface but at its most anterior point It is asymmetrical at first. This asymmetry is clearly expressed in the earlier appearance of the left (anterior) eye The tail blastema is likewise asymmetrical The regenerates illustrate another point of general interest the main direction of outgrowth both of head and of tail blastemas is at first perpendicular to the cut surface and not in the main axis of the old piece Barfurth (1891) was the first to observe this on tail regenerates from oblique sections in amphibian tadpoles and regeneration perpendicular to the cut surface is occasionally referred to as Barfurth s rule In later stages the heads and tails straighten out. Rulon (1936) has interpreted these results on the basis of Child s gradient theory

4. PRODUCTION OF TWO-HEADED PLANARIANS (*DUPLICITAS ANTERIOR*)

EXPERIMENT 55

Remove the head behind the eyes (*a* Fig 38 *c*) and split the anterior two-thirds of the animal in the median line (*n*) The separated parts have a strong tendency to heal together and if necessary the slit must be reopened several times within the next 12–24 hours Note the appearance of new eyes and new pharynges The experiment shows that the regenerate of a *Planaria* behaves like a 2-cell stage of a salamander or of a sea urchin. Each half tends to reconstitute a whole organism (Fig 38 *d*)

5 PRODUCTION OF TWO-TAILED PLANARIANS (DUPLICITAS POSTERIOR)

EXPERIMENT 56

Remove the head behind the eyes and split the posterior two-thirds of the animal Two tails will form if the parts are kept separate by continued reopening of the slit.

6. EXPERIMENTAL PRODUCTION OF BIPOLAR FORMS (POLAR HETEROMORPHOSES)

(After F S Miller)

The previous experiments have given evidence that the polarity is usually maintained in regenerating planarians Cases in which the polarity is changed are therefore of special interest. Morgan (1898) was the first to describe the regeneration of two heads from the anterior and the posterior cut surfaces of a short transverse piece and called this phenomenon "heteromorphosis Later on the term was used to designate all kinds of atypical regenerations for instance the regeneration of antennae in the place of amputated eye stalks in arthropods To avoid confusion inversions of polarity are called "polar heteromorphoses. They occur rather frequently in very short transverse pieces of the post pharyngeal levels. Rustia (1925) has shown that the rate of their occurrence can be controlled and increased by various agents (ether chloretone etc.) F S Miller (1937) succeeded in raising the incidence of bipolar heads very substantially by treatment with strychnine The following experiment is based on her data An interpretation of bipolar forms of *Planaria* on the basis of the gradient theory was given by Child (1915 1941, and others) and by Rustia (1925)

Experiment 57

Material

Dugesia dorotocephala 10–15 specimens per student
other material as on page 166
prepare a fresh solution of strychnine sulphate M/100,000 for each experiment

Procedure

Make all sections in water, not in the strychnine solution For the following experiments only post pharyngeal one-eighth pieces will be used *E* pieces (Fig 38 *e*) give the highest percentage of bipolar heads *F* pieces may also be used Cut in the following order *a d, f e* (Fig 38, *e*) Discard all but the *E* and *F* pieces Transfer these fragments immediately to the strychnine solution cover the dishes and keep them in a dark, cool place After 12–16 hours transfer all pieces to spring or well water A longer exposure is lethal Check the cultures frequently and remove dead fragments Observe the development of bipolar forms Atypical heads (see p 188 Fig 42) will be found occasionally Note the direction of the beat of cilia (cf Rustia, 1925 and Miller 1937) The percentage of bi

polar heads can be increased further by delaying the posterior cut for 12–24 hours. Run parallel cultures of *E* and *F* pieces in well or spring water as controls

7 PRODUCTION OF DUPLICITAS CRUCIATA

(After Silber and Hamburger)

EXPERIMENT 58

Duplicitas cruciata is one of the strangest duplications occurring in animals It is a complete duplication in which two heads and two tails are present. The two heads face in opposite directions and have a common median plane likewise the two tails point in opposite directions However the median plane of the heads and that of the tails are perpendicular to each other (Fig 38, *g*) Duplicitas cruciata in amphibians is discussed on page 75 (*see also* Fig 20)

Material

Dugesia tigrina or *D dorotocephala* 10 specimens per student
other material as on p 166

Procedure

Make a longitudinal cut in the median plane through body and tail to a point behind the auricles (*o–p* in Fig 38 *f*) After 24 hours make a transverse cut (*r*) at a short distance in front of the crotch, so that the two half tails are held together by a narrow connection about one-third their width. If necessary reopen the longitudinal cut. Make 6–10 operations. Check the operated animals on the following days Reopen the median slit if necessary

In a high percentage of cases the anterior transverse surface will regenerate a normal head and another normal head will develop in the crotch (Fig 38 *g*) The crotch heads are occasionally duplicated or abnormal The anterior heads are either normal or in a few cases absent Observe the movements of these monsters after completion of the regeneration. Either head may take the lead with the other head and the tails trailing behind, and they may alternate in leading

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C REGENERATION IN AMPHIBIAN LARVAE

1 TAIL REGENERATION

Tails of urodele and anuran larvae and of adult urodeles regenerate when amputated It is advisable to use larvae because the regeneration in adults takes several months The experiment illustrates the differentiation of blastema into complex structures such as vertebral column spinal cord, musculature blood vessels etc If oblique cuts are made the axis of the regenerated tail will be at first perpendicular to the cut surface (Barfurth s rule [Barfurth 1891] see p 169) Later on the regenerate will straighten out.

The experiment affords no opportunity to study the origin of the blastema cells This question is not yet definitely answered because it is difficult to trace cells *in vivo* The old view that each tissue which is exposed at the cut surface regenerates its own kind holds true in only a few instances Naville (*1922 1924*) in a masterly study has traced the muscles of the regenerated tail to injured muscles at the cut surface which dedifferentiate to a certain degree and then redifferentiate into muscles Other histological and experimental studies give good evidence that most of the blastema cells originate in a different way partly from indifferent mesenchyme cells of the tail stump and partly from old tissue which dedifferentiates completely then forms indifferent cells and eventually redifferentiates possibly along new lines This latter process is called metaplasy (see Schotté 1939)

EXPERIMENT 59

Material

Amblystoma or *Triturus* or *Rana* any species (stages from swimming stages on)

1 pair of fine forceps	Petri dishes
pipettes	chloretone 1 3 000 or MS 222
finger bowls or Lily dishes	1 3 000 *for narcosis*

Procedure

1 Narcotize 10–20 specimens of equal size (take measurements) Operate in narcotic in Petri dishes

2 With a pair of fine scissors amputate the tails In 5 10 specimens make the cuts transversely In 5–10 specimens make them obliquely

Record the details of the operation Make sketches of representative cases

3 Feed the animals daily (pp 21 27)

4 Observe the wound healing and the daily progress of regeneration the appearance of the blastema its outgrowth, the differentiation of the fins, pigmentation etc Observe in particular the direction of outgrowth in the obliquely cut animals The angle between the main axis of the animal and the axis of the regenerating tail can be observed best on the first days after the appearance of the blastema. Later on the regenerating tip straightens out

2. LIMB REGENERATION

Amputation of parts of forelimb or hind limbs of amphibian larvae at any level results in a regeneration of the lost parts Urodeles retain their regenerative power throughout life but anuran legs will not regenerate after metamorphosis Complete regeneration was observed in urodeles even after removal of the girdles Usually the amputated parts are restored completely, but occasionally the regenerate is atypical For instance it may develop fewer digits or toes than normal (hypodactyly) Of special interest are duplications for example the formation of two feet or of supernumerary digits (hyperdactyly) (Weiss, 1925) These cases give evidence that the early regeneration blastema cannot be a rigid mosaic but that it has regulative properties The origin of the blastema cells is not yet definitely established The careful histological studies of Butler (1933) and Thornton (1938) give strong arguments for the view that skeletal and muscle cells at the amputation stump dedifferentiate and then redifferentiate possibly along entirely new lines

PRELIMINARY EXERCISE

In order to find out if the development of the external form of regenerates proceeds along the same lines as does normal limb development study the development of normal forelimbs in *Ambystoma* (any species) Start out with 3–5 larvae of stage H40 and make a complete series of sketches up to stages in which all digits are formed Narcotize the animals during observations Compare with the stage series for *Triturus* by S Glueck sohn (1931) Note in particular the appearance of the elbow the sequence of the appearance of the digits and their relative proportions. Take notes of the rate of differentiation under the conditions of your experiment The animals should be kept in a constant temperature if possible

Material

Ambystoma larvae with 4 digits, 4–6 specimens for each student. Select specimens whose limbs and toes are completely intact. When several specimens are kept in the same dish it happens frequently that they snap and injure one another's limbs particularly if they are not fed adequately It is therefore advisable to rear the specimens to be used in this experiment singly in Lily cups

1 pair of fine scissors
finger bowls or Lily cups
a dish with Permoplast bottom
pipettes
chloretone (1 3 000) or MS 222 (1 3 000) for narcosis

Procedure

1 Narcotize 4–5 specimens

2 Make a sketch of the extended right arm of a representative specimen Use the camera lucida if available In order to mount the arm horizontally use a dish with Permoplast bottom Make a deep groove in which the body of the animal fits and spread the arm over the edge of the groove

3 In 2–3 specimens make a transverse cut through the right humerus (Fig 39 *a*), in 2–3 specimens amputate the right hand through the wrist (*b*) Use a pair of fine scissors Indicate the level of cutting on the sketch

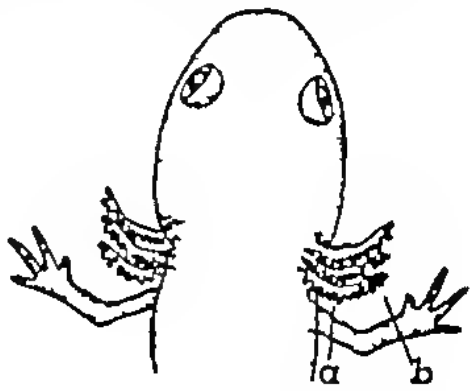

FIG 39 —Forelimb regeneration in salamander larvae *a b*—levels of sectioning (see text)

4. Give each specimen a serial number and place each in a separate dish. Rear in a constant temperature if possible

5 Follow the regeneration for several weeks Make a complete series of sketches Compare the regeneration with normal development Note that in the specimens amputated at the humerus the digits will appear first and the parts between the digits and the cut level will be restored later If time permits both the amputated ends and the fully regenerated limbs should be fixed and stained with methylene blue (after Lundvall p 134) in order to find out if the cartilaginous skeleton has been restored completely

3 THE WOLFFIAN LENS REGENERATION IN URODELA
(After H Wachs 1914)

The lens regeneration is unique among the regeneration phenomena G Wolff (1895 1901) discovered that if the lens is carefully extirpated

in a urodele, a perfectly normal oew lens will be formed Strangely enough the origin of the regenerated lens is different from that of the embryonic lens in that the upper margin of the iris, and not the epidermis forms the regenerate (Fig 40, *c–e*) This case illustrates an important principle that the same end result can be achieved by two entirely different modes of development The lens regeneratioo is unusual in other respects It is the clearest case of "metaplasy' known so far (see p 173) the differen tiated aod pigmented cells of the iris undergo a dedifferentiatioo and a subsequent redifferentiatioo into highly specialized lens cells (Wachs, 1914, Sato, 1930 and others)

If the operatioo is done carefully the upper iris does not suffer any in jury This observation raises an important issue. In most instances of re generation the creatioo of a wound surface is considered essential for the initiation of the regeoeratioo process In the present instance the neces- sary stimulation for the onset of regeoeratioo must be provided by other factors Spemann (1905) set forth the hypothesis that the retina exerts an inductive effect oo the upper iris, probably by releasing an inductive substance into the posterior chamber In the normal undisturbed eye a chemical substance emanating from the normal lens inactivates or in hibits this retina factor If the lens is removed the balance between the "retina factor and the 'lens factor' is upset and the retina factor exerts its influence on the iris A number of ingenious experiments by Wachs Sato and others have confirmed this hypothesis (reviews in Wachs 1919, Mangold 1931 Spemann 1938)

Lens regeneration in *Anura* has been reported in only a few instances it is definitely limited to larval stages Anurans should not be used for this experiment. Of the urodeles all European *Triturus* species and the Japanese *Tr pyrrhogaster* are capable of Wolffian regeneration throughout life Data oo *Ambystoma* are scarce. Ballard (1936) in a short note re ported positive results only for young larvae of *A maculatum* and *A ti- grinum* up to stage H43 in later larval stages these forms as well as *A microstomum A jeffersonianum*, and *A opacum* showed oo regenera tioo Stone and Dinnean (1940) found no Wolffian regeneration in *A maculatum* in stage H46 and earlier

In our class experiments regeneration was found to occur in *A opacum* and *A maculatum* larvae of stage H46 and older But the number of suc cessful cases was variable and oever exceeded 50 per cent. Likewise the rate of regeneratioo varied greatly from 10 days to over 3 weeks

We recommeod using only young larvae and making a considerable oumber of experiments It will be found that after some practice a large oumber of operations can be done in a short period

Lens regeneration occurs regularly in *Tr torosus* (personal communication of Mr D Bodenstein)

Material

swimming larvae of *Tr pyrrhogaster* or *Tr torosus* or *A opacum* or *maculatum* stage H43 and older 6–10 specimens for each student

a straight glass needle (Fig 40, *a*), which should be shorter and much stronger than a needle used for early embryos. It should not be elastic and should taper into a fine point in order to pierce the tough cornea.

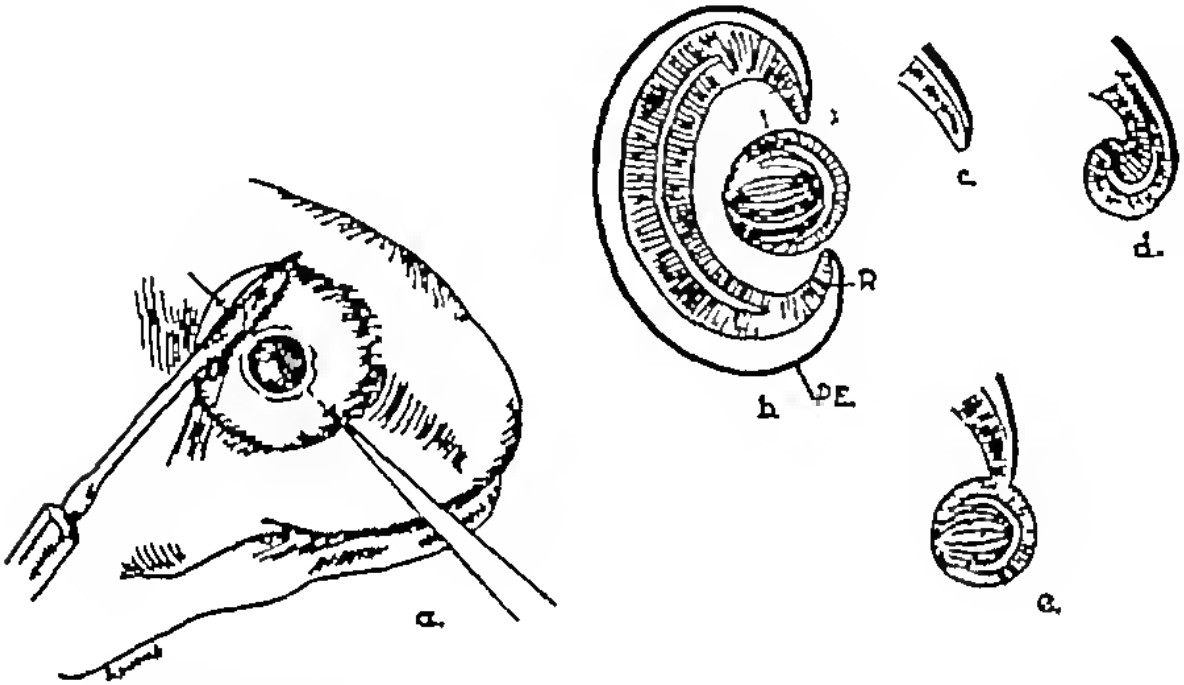

FIG. 40 —The Wolffian lens regeneration *a* = operation (see text) *b* = normal eye with lens *c–e* = the process of regeneration from the upper ris = dotted lines in *b* (see text) *P E* = pigment epithelium *R.* = retina

iris knife	insect pins
dishes with Permoplast bottom	chloretone (1 3 000) or MS 222 (1 3 000) for narcosis
finger bowls or Lily cups	a camera lucida is desirable
filter paper	

Procedure

1 Prepare a groove in the Permoplast dish in which the larva fits when lying on its left side

2 Narcotize 6–10 larvae

3 Fasten a larva right side up in the Permoplast groove using wide strips of filter paper and insect pins

4 Make a slit in the cornea above the lens slightly longer than the diameter of the lens. Proceed as follows (Fig 40 *a*) Carefully pierce the cornea with the needle and holding it horizontally push it along the inner

surface of the skin and out again Carefully avoid any injury to the eye or lens Scrape gently with the iris knife against the skin over the needle until the skin is cut

5 Lift the lens out intact Work the tip of the needle carefully between the iris and the lens and use the needle as a lever Do not pierce the lens In a successful operation the entire spherical lens sticks to the needle and can be seen easily, although it is glass-clear Avoid hemorrhages Discard specimens in which the retina has been injured and operations *in which you have not seen the intact lens after extirpation*

6 Take a protocol

Repeat the operation on at least 6 animals

7 During the following weeks feed the animals frequently Make observations about twice a week. Narcotize the animal each time. Observe the collapse of the pupil after the operation and its gradual reopening The details of the regeneration cannot be seen without sectioning, but the width of the pupil gives an indication of the size of the regenerating lens. Make sketches. Observe the gradual clearing up of the cornea, which became opaque after wound healing, and the gradual widening of the pupil

The structural changes which occur, meanwhile, in the upper iris are illustrated diagrammatically in Figure 40, *c–e*

8 After 3–4 weeks narcotize all the animals and fix them in 10 per cent formaldehyde After a few minutes the lenses will become opaque and thus very distinct. Wash the animals in water Mount them in the Permoplast groove and dissect and peel off the skin from over both eyes The left eye serves as a control Make camera lucida drawings of both eyes and lenses. For this purpose place the animal in the Permoplast groove so that first the right lens and then the left lens is in exactly the same plane Calculate the ratio lens diameter eye diameter for both eyes and obtain thus a quantitative estimate of the degree of regeneration

If time permits and facilities are available, a number of experimental animals should be sacrificed at 5–25 days after operation and sectioned to obtain early stages of regeneration However the results are unpredictable (at least in *Amblystoma*) on account of the high individual variation in the speed of regeneration

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PART V

THE GRADIENT THEORY

A. GENERAL REMARKS

The gradient theory of C M Child is an ingenious attempt to interpret development and reconstitution under a uniform concept All structural differentiations, in the embryo as well as in the regenerating adult body in asexual reproduction of invertebrates as well as in the buds of plants are considered as expressions of basic physiological activities which precede these structural differences These activities follow definite gradations of intensity the anterior end of an animal the animal pole of an egg or the apical end of a growing bud usually show the highest metabolic activity and the decrement of intensity usually follows the main axis (axial gradients) Similar gradients may be present in dorsoventral and in mediolateral directions. The existence of axial gradients has been demonstrated by Child and his associates by several different methods Foremost among them is the method which makes use of the differential susceptibility' of living tissues to toxic agents If a planarian or a chick embryo is placed in a lethal concentration of potassium cyanide the heads disintegrate first, and the further breakdown of tissue proceeds along the anterior posterior axis (Fig 41) A high degree of susceptibility is considered as an indication of high physiological activity Any number of toxic agents can be used they all tend to show the same basic pattern of physiological activity for a given stage Another method is that of differential reduction or oxidation of vital dyes

The nature of the physiological activities of which susceptibility etc are indicators is obscure According to Child the underlying chemical processes may be different in different animals and plants and of a different nature even in the anterior posterior dorsoventral and mediolateral gradient systems of the same organism Therefore Child uses the noncommittal terms axial gradients or activity gradients

The implications of this theory for the problem of determination are far reaching According to the gradient theory the fate of a given embryonic or regenerating group of cells is primarily determined by its relative position in a gradient system For instance in a transverse piece of a planarian the area of highest physiological activity will give rise to head structures and the area of lowest physiological activity will give rise to tail structures the intermediate levels of activity will be correlated with trunk structures, like pharynx (the relations of levels of activity and structures are, of course relative and not absolute values) If in a very short

transverse piece of a planarian, both the anterior and the posterior cut surfaces are approximately on the same metabolic level, then both will regenerate heads (p 170) The "head frequency experiment (Expt 64) has been designed to demonstrate that the relative position of a cut surface on the main axis plays a role in the determination of the regenerating head High levels give rise to a higher percentage of normal heads than do posterior levels, and a quantitative relation can be established between the frequency of head abnormalities and the position of the regenerating surface on the anterior posterior axis

In a further elaboration of the gradient theory Child has introduced the concepts of 'physiological dominance "subordination,' "physiological isolation, etc. and has reinterpreted organizer and inductor activities duplications individuation etc., in terms of the theory His books (see also the complete bibliography of Child s publications in Hyman and Van Cleave, 1938) are recommended for collateral reading Critical evaluations of the theory will be found in Parker (1929), Needham (1931), and Spemann (1938) One of the major criticisms is that the measured differentials of "physiological activity may he the effects rather than the cause of local structural differentiations The difficulties involved in this controversy lie in our ignorance of the chemical or physiological processes underlying the ' metabolic gradients Apart from these criticisms, the axial gradients must be given due consideration as important factors in the complex of conditions which determine symmetry relations and structural differentiations of embryonic cells

B EXPERIMENTS

1 THE SUSCEPTIBILITY GRADIENT IN PLANARIA (KCN)
(After Child)

The term ' susceptibility gradient has been discussed in the preceding section *Planaria* has been used widely for a demonstration of disintegration gradients. The following experiment was devised by Child (1913)

EXPERIMENT 62

Material

Dugesia (*Planaria*) *dorotocephala*, 15–30 specimens for each student
small brush for handling planarians
M/500 potassium cyanide (130 mg in 1 000 cc. of spring or tap water)
M/1 000 potassium cyanide (65 mg in 1 000 cc of spring or tap water)

Note —The solutions must be prepared immediately before use. KCN is poisonous. Be very careful to avoid spilling it on the table or on the microscope

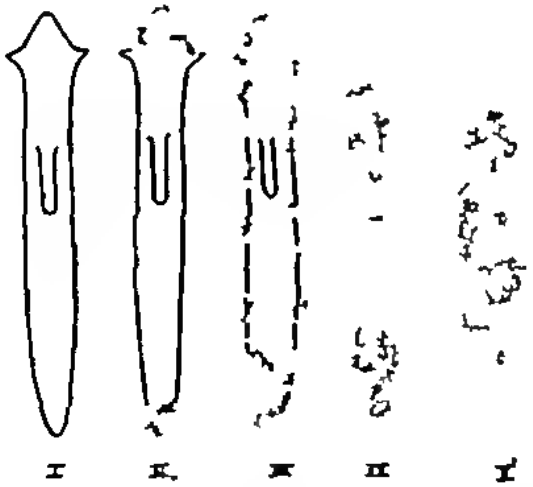

FIG 41 —Stages of disintegration of *Planaria* (see text) (from Adams, 1941)

Procedure

Fill one dish completely with the stronger solution another one with the weaker solution and place 8–15 animals in each dish Cover the dishes with glass plates excluding all air bubbles Observe the animals under the low power of the binocular microscope for a period of from 1 to several hours. Child distinguishes 5 stages of disintegration (Fig 41) Tabulate your results indicating the time required for the different stages Adams

(1941) suggests assigning the following arbitrary values to each stage I=40 II=30 III=20, IV=10, V=0 and calculating the total value for a given time of observation in the following way Multiply the value of a stage by the number of worms at that stage add these figures up and di vide the total by the number of worms used Make two graphs one for disintegration in M/500 KCN the other for disintegration in M/1,000 KCN Plot the time on the abscissa and the disintegration values on the ordinate Compare the two disintegration curves

Note —The time required for complete disintegration varies consider ably for different lots of material, for different temperatures etc It is ad visable to run a test experiment with the solution to be used in the class If necessary, start the class experiment before the laboratory period be gins

Further suggestions —Disintegration experiments of the same type have been made on a large number of animals and with a large number of toxic agents Further experiments may be selected from the literature which is listed in Child (1941, Appen III, p 734)

2. OXIDATION REDUCTION GRADIENTS IN THE CHICK EMBRYO (JANUS GREEN)

(After Rulon)

Physiological axial gradients can be demonstrated by the differential reduction of vital dyes by living tissue For instance the blue oxidized form of methylene blue will be reduced to the colorless leuco form and Janus green will change to pink under conditions of low oxygen tension The decoloration proceeds in anterior posterior direction, thus indicating an axial gradient of differential oxygen need If older stages are used different organ primordia will stand out as areas of high oxygen require- ment and complex patterns of differential reduction capacity rather than simple gradients will become apparent (for details see Rulon, 1935)

EXPERIMENT 63

Material

chick embryos any stage between 5 and 20 somites 4–5 embryos for each student

hair loop
1 pair of fine scissors
watchmaker forceps
scalpel
wide mouthed pipette
medicine droppers
heating plate
depression slides and cover glasses
melted vaseline for sealing of cover glasses
watch glasses with cotton nests (p 134)
filter paper

c 9 per cent sodium chloride (Keep at 35°–40° C over a Bunsen burner or a heating plate)

1/50 000 oxidized Janus green made up in 0 9 per cent salt solution (Keep at 35°–40° C)

Procedure

1 Place an egg on a cotton nest break open the shell on top and expose the embryo without injuring it

2 Cut out the blastoderm at a considerable distance from the embryo using a pair of fine scissors Transfer the embryo to the depression slide with saline solution using a wide mouthed pipette Carefully avoid any injury to the embryo

3 Flatten the blastoderm in the following way Cut out a ring of filter paper with a central hole of about the diameter of the area pellucida. Flatten the blastoderm by manipulating it with the hair loop withdraw most of the saline solution and place the ring on the flattened blastoderm so that the embryo proper is exposed In this way the embryo is prevented from rolling up

4 Add the Janus green solution immediately Stain the whole embryo for 8–20 minutes (times are different for different stages) After it is thoroughly stained blue-green withdraw the dye and wash the embryo with saline

5 Add salt solution cover the depression slide and seal the cover glass with vaseline

6 Return the embryo to the incubator or preferably place it on a heating plate Observe the gradual change of color to brilliant red Make a series of sketches of the color change indicating the gradient of oxygen requirements Consult Rulon (1935) for all details for instance the structures which change first in different stages times required for reduction etc

3 THE INFLUENCE OF THE AXIAL GRADIENT ON HEAD REGENERATION IN PLANARIA

(After Child and Watanabe 1935)

HEAD FREQUENCY (h.fr)

Child has shown that in short transverse pieces of *Planaria* head regeneration is not always complete The head regenerates can be arranged in a graded series ranging from normal heads to small outgrowths These were classified by Child in five arbitrary groups (see Fig 42) I Normal. II Teratophthalmic (abnormal eyes) The shape of the head is almost normal but the eyes show all degrees of approximation and fusion to the

cut equally Do not use the pharynx as a landmark If the pharynx is extruded, remove it altogether

Repeat the experiment with 10–14 other animals.

6 Transfer all anterior one sixth pieces to the dish labeled A all median pieces to dish B, and all posterior pieces to dish C. Take a protocol Place the dishes in a cool dark place

7 After 2 days remove the dead fragments Do not feed

8 Study the regenerates 10–14 days after operation Inspect each lot separately Make sketches of representative specimens of the 5 head types If not all are represented in your material, exchange specimens with your neighbors Determine the numbers of the different head forms in each lot and tabulate the results in the following table Add the total figures for the class and plot them as a curve

TABLE 6

Number Operated	Date	Head Forms						Results of Class	
		I	II	III	IV	V	Head Frequency	Number Operated	Head Frequency
A									
B									
C									

EVALUATION

1 Compare head frequency in C-pieces with that in tail pieces of Experiment 51 (p 167) The only variable in the two experiments is the length of the piece What conclusion can be drawn?

2 Compare head frequency in pieces A, B and C of the present experiment. The only variable is the body level What conclusion can be drawn?

4 DELAYED POSTERIOR SECTIONS

(After Child and Watanabe)

Note —This experiment should be made together with Experiment 64

This experiment gives indirect evidence that the formation of abnormal heads in short transverse pieces is controlled by an inhibiting agent which is released at the posterior cut surface shortly after the cut is made It is assumed that this inhibiting agent travels in posterior anterior direction along the ventral nerve cords and exerts its influence directly on the head blastema

Experiment 51 (p 167) has shown that level *c* forms normal heads provided that no other cut is made posterior to *c* If such a cut is made in *d* (Expt. 64) then abnormal heads appear the head frequency is lowered It is possible to determine approximately how much time is required for the posterior depressing' agent to reach the head blastema and for how long a period the head blastema is susceptible to this inhibiting influence This is done by delaying the posterior cut *d* for a series of time intervals It was found that the inhibitor is effective only within the first 8–12 hours after onset of the head regeneration in *c* If the posterior cut is delayed longer then the head frequency is near 100 per cent

Experiment 65

Material

Dugesia (*Pl*) *dorotocephala* or *D tigrina* 15–30 specimens for each student (Select individuals of uniform size)

other material as in Experiment 64

Procedure

1 Cut all specimens in the *c*-level (Fig 38 *a*) and discard all anterior pieces

2 Make cuts in level *d*

a) in 5–10 specimens 2 hours after cut *c*

b) in 5–10 specimens 8–14 hours after cut *c*

c) in 5–10 specimens 24 hours after cut *c*

Discard the posterior pieces Keep the 3 lots in different dishes and handle the material as in Experiment 64

3 After 2 weeks calculate the head frequency for the 3 series as in Experiment 64 Tabulate and plot the results on graph paper Compare your curves with those of Child and Watanabe (1935) for *D dorotocephala* and with Watanabe (1935) for *D tigrina* Note that these authors worked with one-eighth and one tenth pieces. Also temperature etc have an effect on the results

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APPENDIX

PLAN FOR A ONE SEMESTER COURSE

As was stated in the Introduction the experiments are not arranged in a definite sequence in which they should be scheduled No experiment or technique is based on any experience gained in a preceding experiment. The choice and arrangement of the experiments is left to the discretion of the instructor To facilitate the organization of a one semester course, we give the schedule which we followed approximately but with many variations from year to year

Normal development of *Amblystoma* or *Rana*
Development of behavior (Expt. 40)
Development of embryos in narcosis (Expt. 41)
Vital staining of amphibian embryos (selections from Expts. 1–8 according to stages available)
Balancer and limb transplantations (Expt. 14–16)
Artificial parthenogenesis (Expt. 9)
Cleavage under pressure (Expt. 10)
Duplications by inversion of eggs (Expt. 11)
Partial limb and eye extirpations (selection from Expts. 18–21 and 30–31)
Production of double hearts (Expt. 22)
Lens induction (selection from Expts. 32–34)
Parabiosis (Expt. 37)
Tail and limb regeneration in amphibian larvae (Expts 59–60)
Lens regeneration in salamander larvae (Expt. 61)
Regeneration in planarians (selection from Expts. 51–58)
Axial gradients and head regeneration (Expt. 63)
Susceptibility gradient in planarians (Expt. 62)
Vital staining of the chick blastoderm (Expt. 42–44)
Chorio-allantoic grafts in the chick (Expt. 45–46)
Transplantation of prospective melanophores in the chick (Expt. 50)

Emergencies cannot be entirely avoided in a laboratory course which uses living embryonic material exclusively They can be met easily by using fixed material of normal embryos and larvae for dissections with a glass needle Also living planarians can be supplied by the large supply houses almost all year around

In the following list the experiments are arranged in three groups according to the skill required to perform them

Easy Experiments 5–10 12 13 18–21 23 29, 34, 37–41 51–60, 62 64, 65
More difficult but suitable as classroom experiments Experiments 1–4, 14, 15 17 22 30–33 42–46, 50, 61 63
Difficult requiring considerable experience Experiments 11 16 35 36 47–49

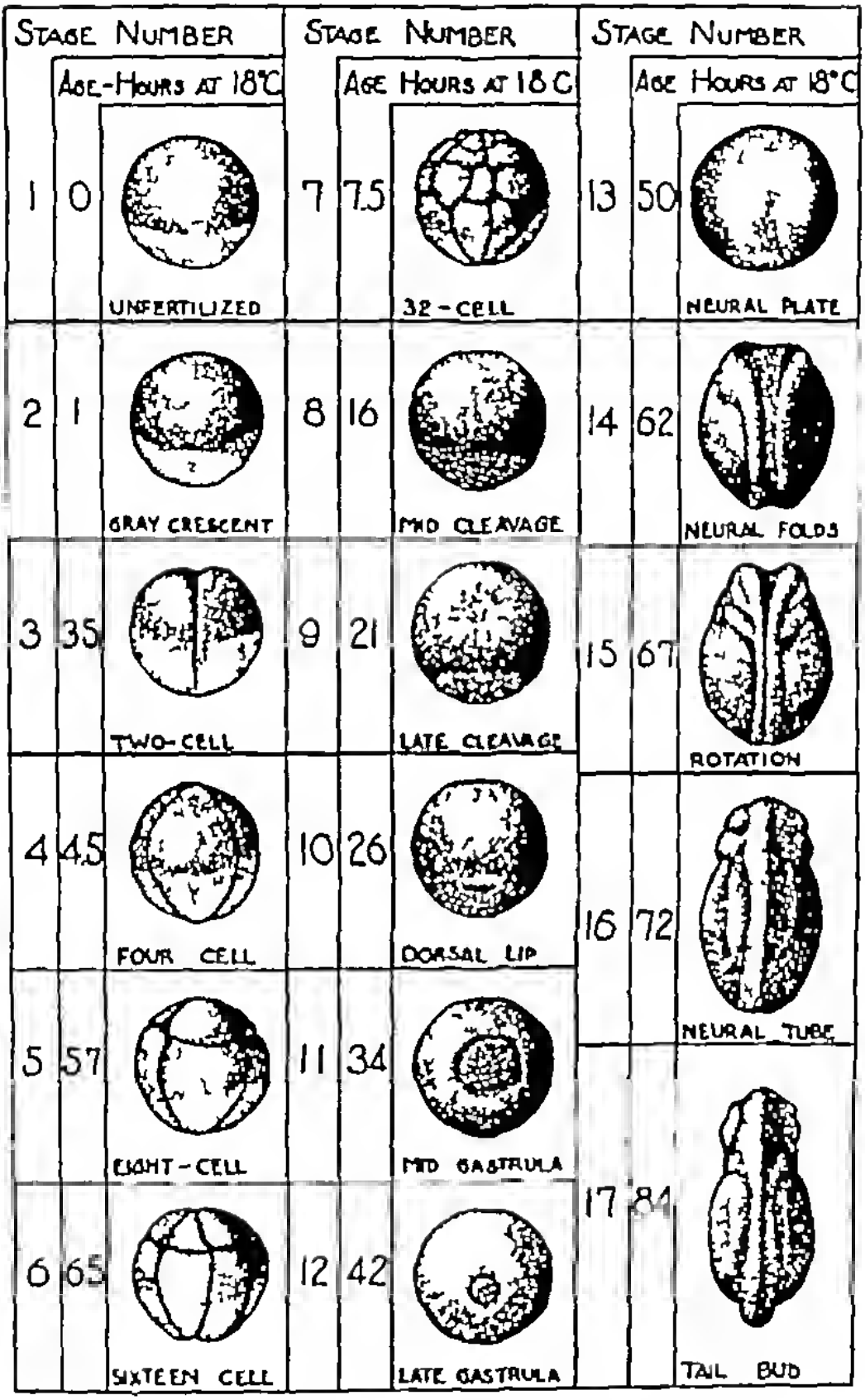


FIG 43a.—Stage series of *R. pipiens* (from Shumway 1940, Anat. Rec 78:139)

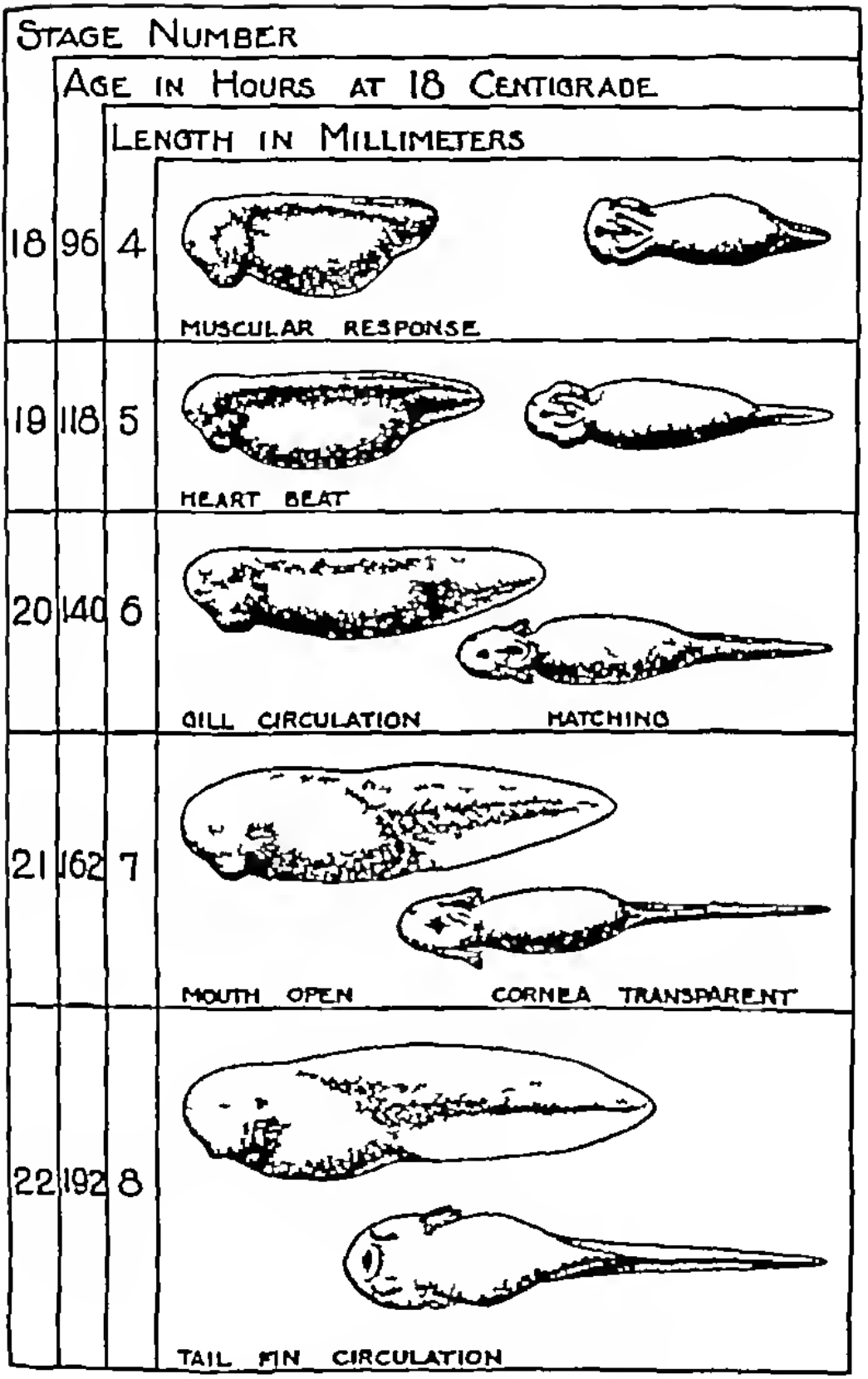


FIG. 43b —Stage series of *R. pipiens*—*cont. and*

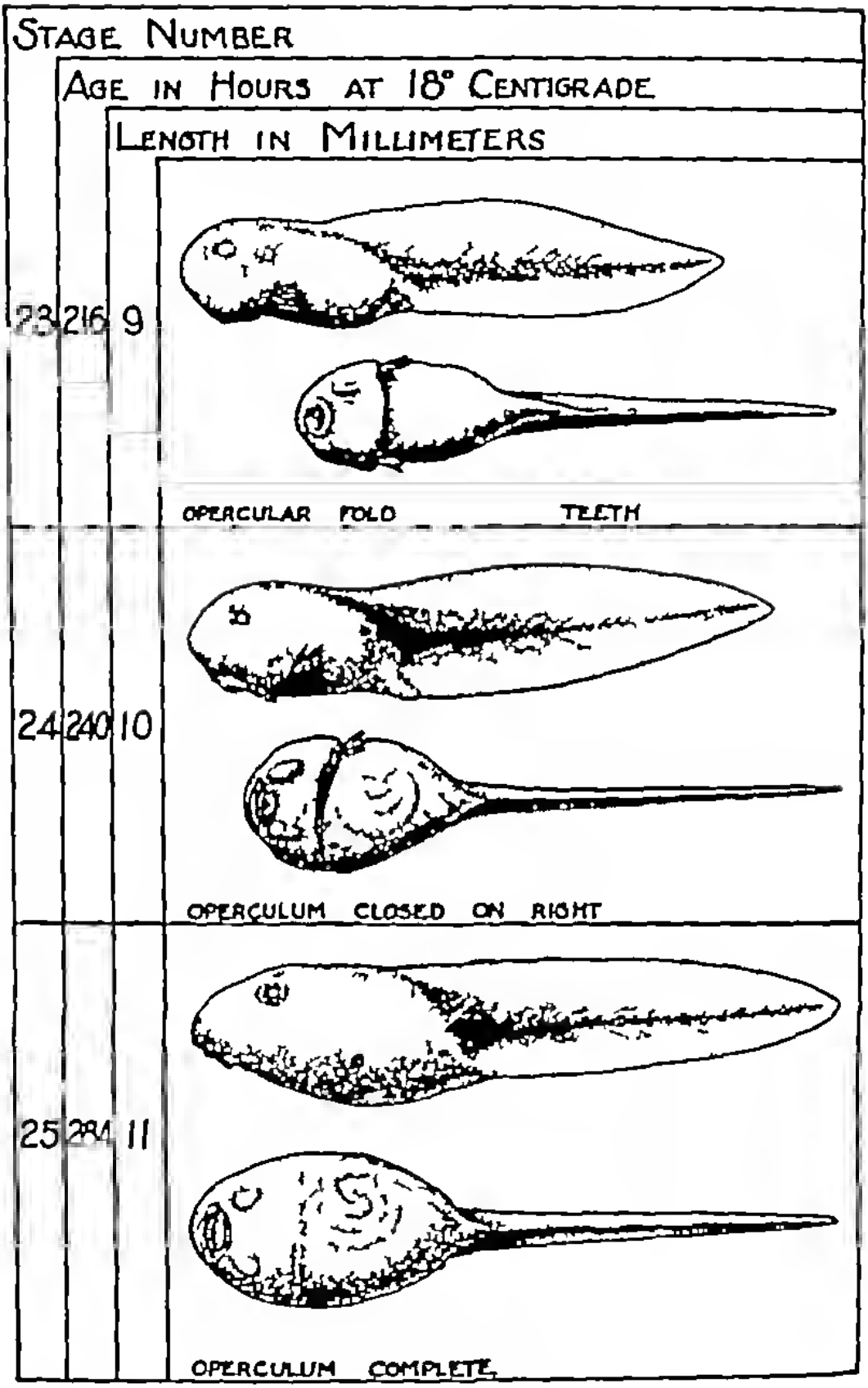


FIG 43c —Stage series of *R pipiens*—continued

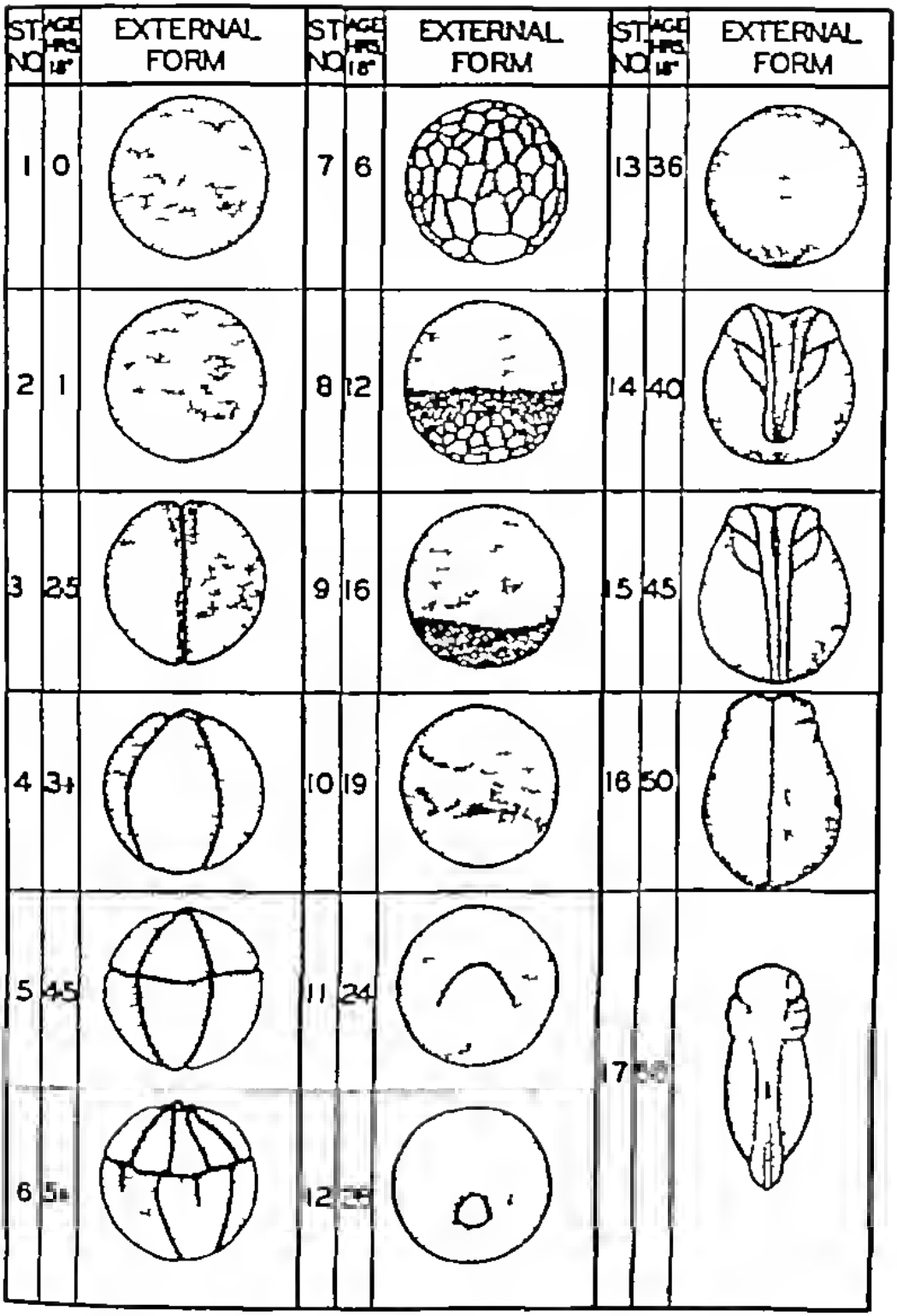


FIG 44a —Stage series of *R sylvatica* (from Pollister and Moore, 1937 Anat. Rec., 68 489)

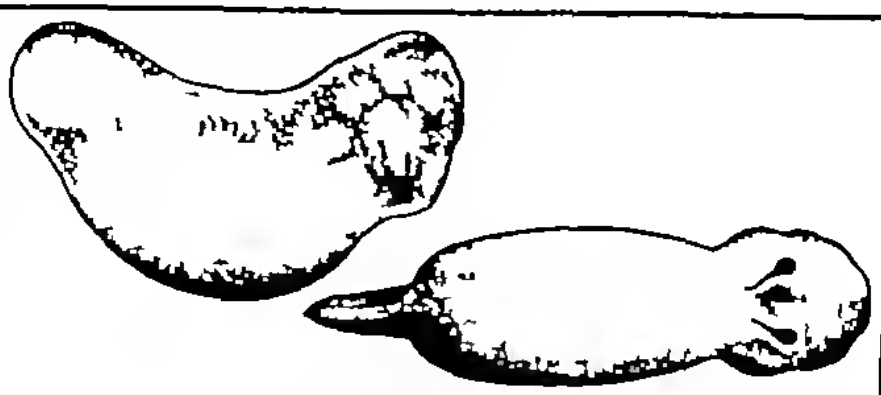
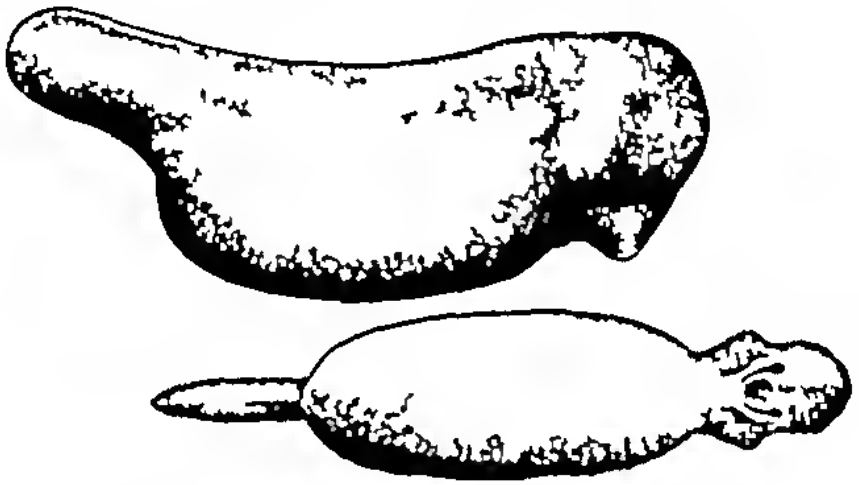
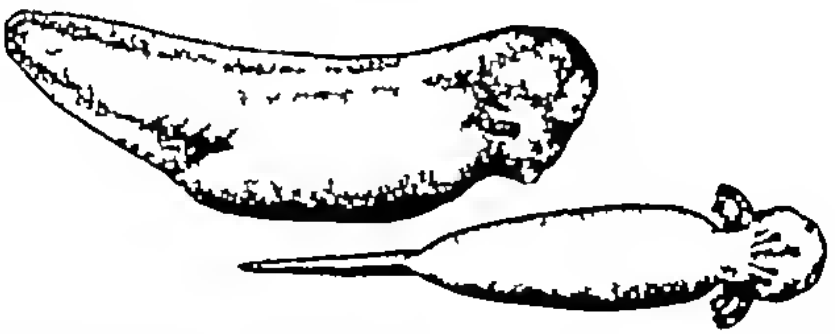
ST NO	AGE HRS		EXTERNAL FORM
18	65	5	 MUSCULAR MOVEMENT
19	75	6	 HEART BEAT
20	90	7	 GILL CIRCULATION SWIMMING — HATCHING

FIG. 44*b* —Stage series of *R. sylvatica*—*continued*

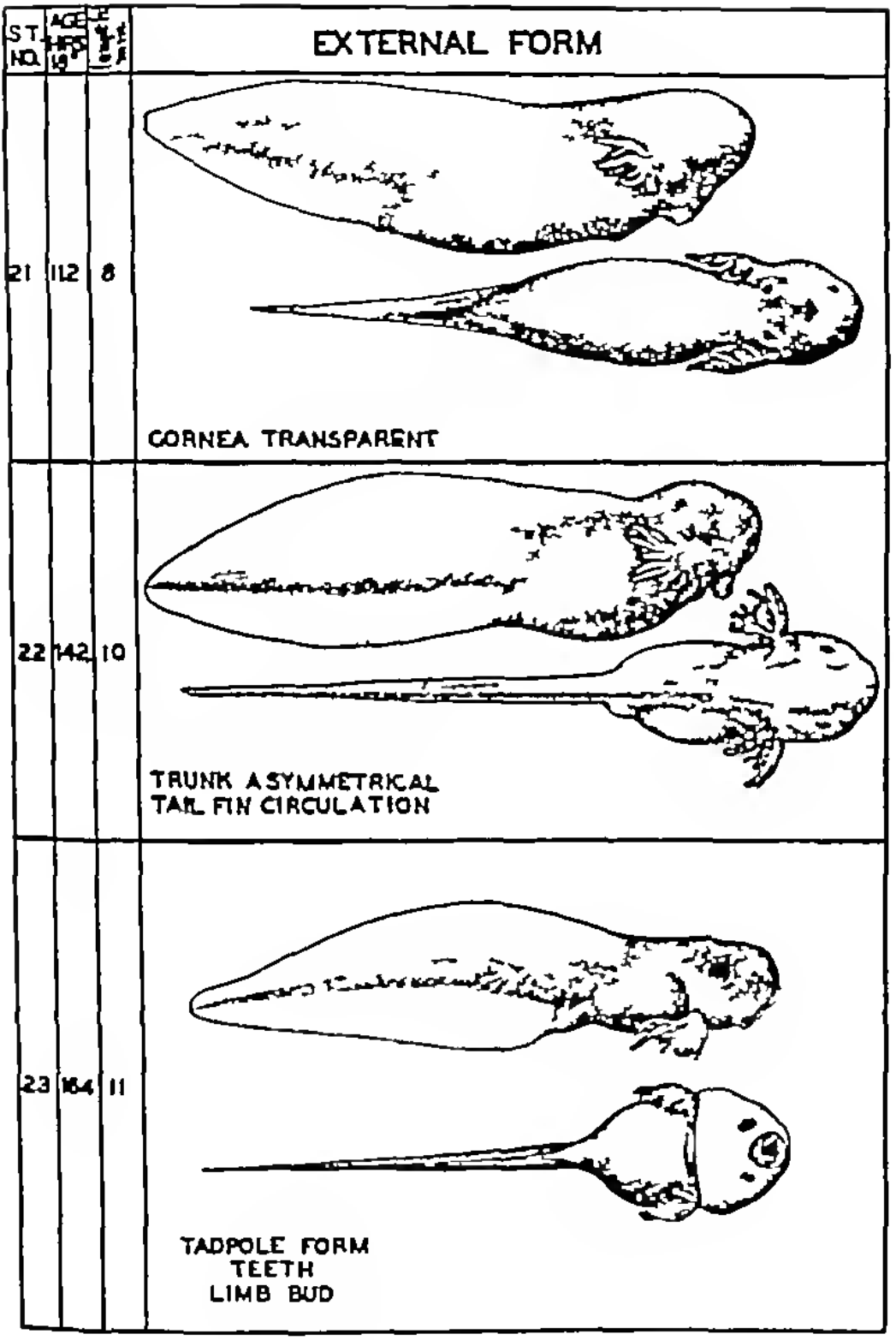


FIG. 44c —Stage series of *R. sylvatica*—*continued*

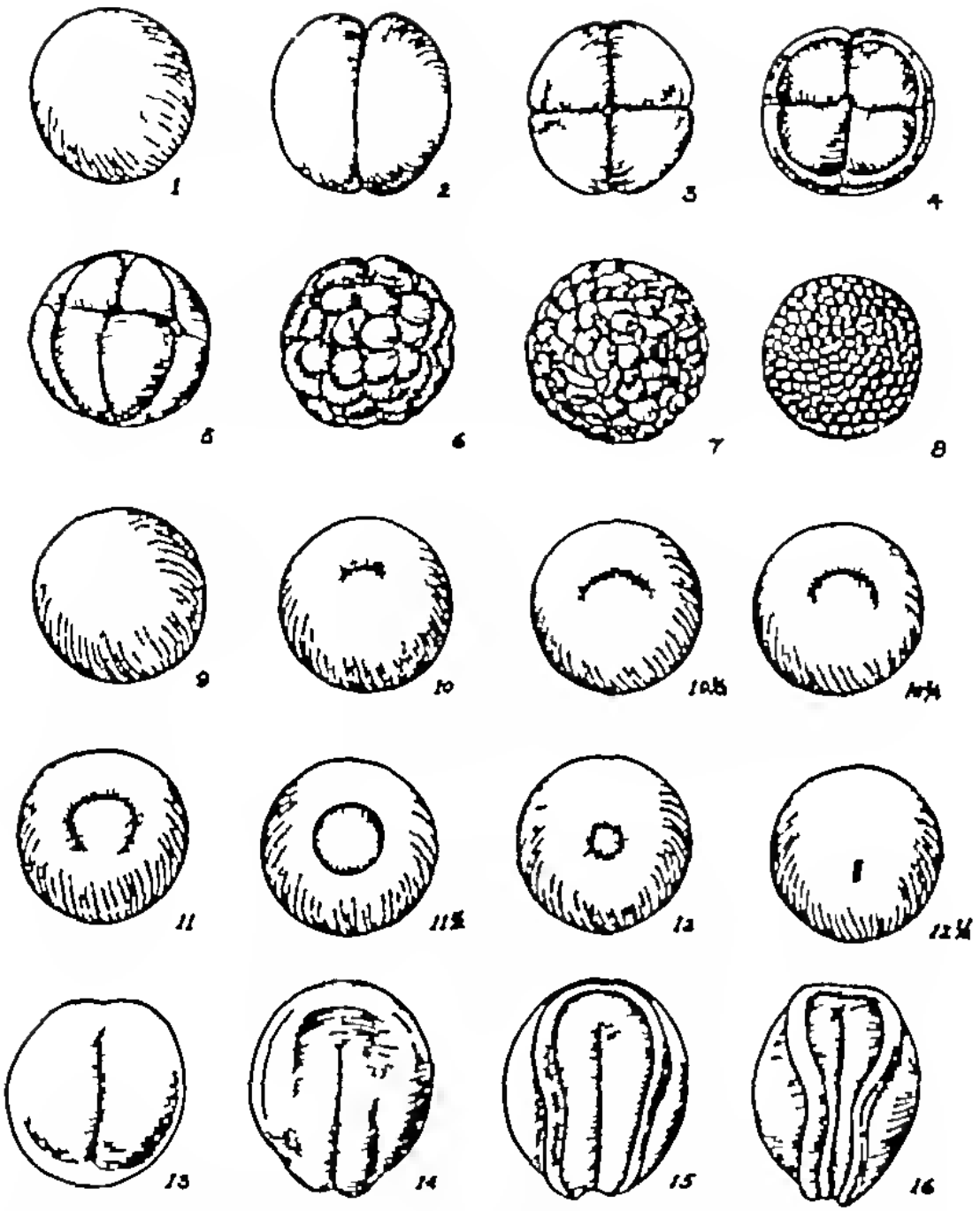


FIG. 45a —Stage series of *A maculatum*. The numbers indicate the stage numbers (After unpublished photographs of Dr R. G Harrison, with permission of the author Drawings by Miss S E. Schweich.)

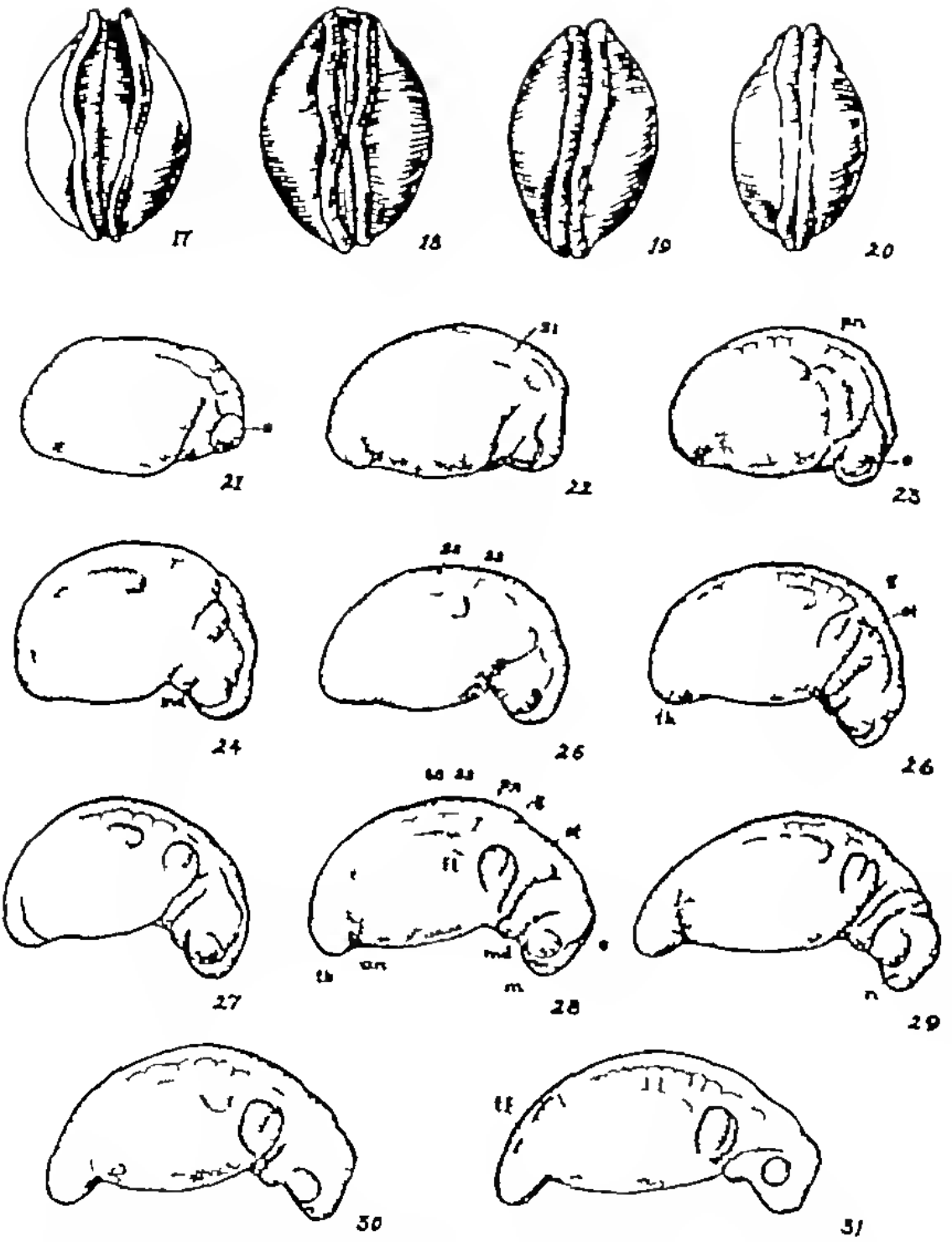


FIG. 45*b*.—Stage series of *A. maculatum*—*continued* *an* = anus *e* = eye *g* = gills *m* = mouth *ma* = mandibular arch *n* = nose *o* = optic vesicle *ot* = otocyst *pn* = pronephros *s* = somites *tb* = tail bud *tf* = tail fin.

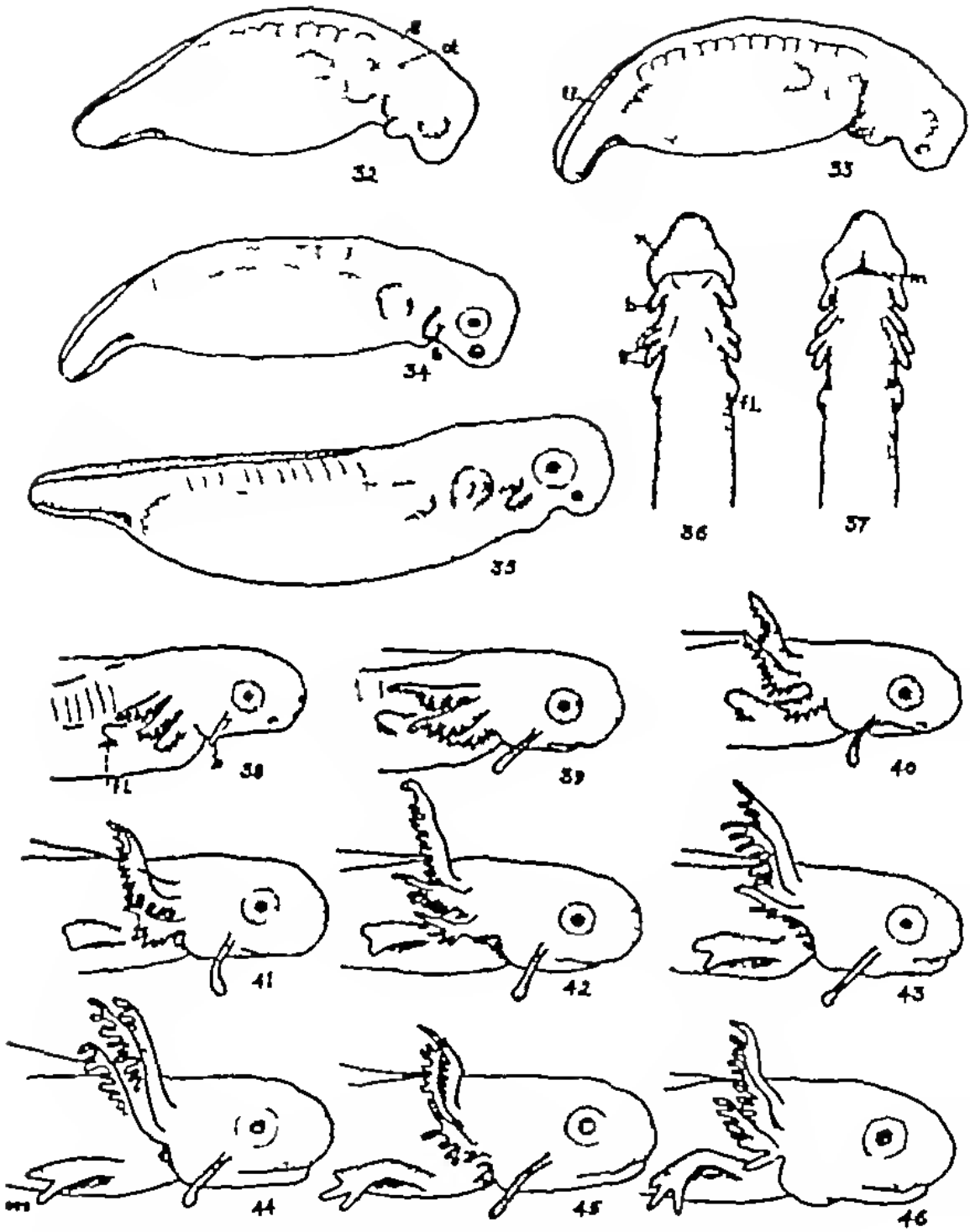


FIG 45.—Stage series of *A. maculatum*—*continued* *b* = balancer *fl* = forelimb *g* = gills *m* = mouth *n* = nose *ot* = otocyst *tf* = tail fin.

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INDEX

(A = Amphibians Ch = Chick Pl = Planarians = illustrated)